A NOVEL SELECTIVE INHIBITOR FOR PLASMA MEMBRANE CALCIUM ATPase 4 IMPROVES VEGF-MEDIATED ANGIOGENESIS

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Research Institute in Healthcare Science

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DECLARATION

This work or any part hereafter has not previously been presented in any form to the University or to any other body whether for the purposes of assessment, publication or for any other purpose (unless otherwise stated). Save for any express acknowledgments, reference and/or bibliographies cited in the work, I can confirm that the intellectual content of the work is the result of my own efforts and no other person.

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Abstract

Ischaemic cardiovascular diseases are the leading cause of death worldwide. Therapeutic angiogenesis provides a valuable tool to treat these conditions by stimulating the growth of new blood vessels in the ischaemic tissue. The pro-angiogenic factor VEGF is the most potent inducer of angiogenesis, and exogenous delivery of VEGF has been a key element of therapeutic strategies. Unfortunately, VEGF-based pro-angiogenic procedures have produced only limited patient benefit. Failure to restore efficient VEGF activity remains a major problem.

VEGF-mediated activation of the calcineurin/NFAT signalling pathway has been identified as a crucial regulator of angiogenesis. Our laboratory has recently shown a novel role for the plasma membrane calcium ATPase 4 (PMCA4) protein as a negative regulator of VEGF-induced angiogenesis via interaction with calcineurin. The recent identification of aurintricarboxylic acid (ATA) as a selective inhibitor of PMCA4 prompted us to hypothesise that inhibition of PMCA4 with ATA should enhance VEGF-induced angiogenesis.

Here, we show that treatment of endothelial cells with nanomolar concentrations of ATA notably enhances calcineurin/NFAT signalling by disrupting the PMCA4/calcineurin interaction. ATA mediated inhibition of PMCA4 results in a significant increase in endothelial cell motility and *in vitro* and *in vivo* blood vessel formation. Low concentrations of ATA do not have any deleterious effects on the viability of endothelial cells or zebrafish embryonic development. However, high ATA concentrations impaired endothelial cell viability, and were associated with toxicity in zebrafish embryos.

This study highlights the potential of targeting PMCA4 to improve VEGF-based pro-angiogenic therapeutic strategies. This goal will require the development of refined versions of ATA without associated toxicity, or the identification of novel PMCA4 inhibitors.

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List of abbreviations

Ad- Adenovirus

ADP - Adenosine Diphosphate

AID- Auto-Inhibitory Domain

AKAP79- A Kinase Anchoring Protein 79

AMD- Age-Related Macular Degeneration

APS- Ammonium Persulfate

ATA- Aurintricarboxylic acid

ATP- Adenosine Triphosphate

ATPase- Adenosine Triphosphatase

BBB- Blood Brain Barrier

bFGF- Basic Fibroblast Growth Factor

BSA- Bovine Serum Albumin

CaM- Calmodulin

CaMBD- Calmodulin Binding Domain

cAMP- Cyclic Adenosine Monophosphate

CASK- Calcium/Calmodulin Dependent Serine Protein Kinase

cDNA- Complementary Deoxyribonucleic Acid

CHP- Calcineurin Homologous Protein

CLI- Critical Limb Ischaemia

Cn A- Calcineuirn A

Cn B- Calcineurin B

CnBD- Calcineurin B Binding Domain

CNS- Central Nervous System Functions

CsA- Cyclosporin A

Ct- Cycle Threshold

DAF-FM- Diacetate 4-Amino-5-Methylamino-2',7'-Difluorofluorescein

DKO - Double Knock Out

DMEM- Dulbecco's Modified Eagle Medium

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribonucleic acid

DSCR- Down Syndrome Critical Region

DVT - Deep Vein Thrombosis

EC- Endothelial Cell

ECGM- Endothelial Cell Growth Medium

ECM- Extracellular Matrix Components

EDTA- Ethylenediaminetetraacetic acid

EGFR - Epidermal Growth Factor Receptor

eNOS- Endothelial Nitric Oxide Synthase

Erk- Extracellular Signal Regulated Kinase

ETCs- Endothelial Tip Cells

FBS- Fetal Bovine Serum

FDA - Food and Drug Administration

FK506- Tacrolimus

FKBP12- FK506 Binding protein

Flk-1- Foetal Liver Kinase-1

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

H^+/K^+-ATPase- Hydrogen-Potassium ATPase

HEK293A- Human Embryonic Kidney 293A Cells

HGFR- Hepatocyte Growth Factor Receptor

HRP- Horseradish Peroxidase

HUVECs- Human Umbilical Vein Endothelial Cells

ICAM- Intracellular Adhesion Molecule

ID4- 3xFlag-PMCA4b(428-651)

Ig- Immunoglobulin

IP- Immunoprecipitation

KDR – Kinase Insert Domain Receptor

La^{3+}- lanthanium

LacZ-

MAGUK- Membrane Associated Gunylate Kinase

MAPK- MAP kinase

MAPK- Mitogen Activated Protein Kinase

MAPKK- MAP Kinase Kinase

MAPKKK- MAP Kinase Kinase Kinase

MCIP1- Myocyte Enriched Calcineurin Interacting Protein 1

MLECs- Mouse Lung Endothelial Cells

MMPs- Matrix Metalloproteinases

MOI- Multiplicity of Infection

mRNA- Messenger Ribonucleic Acid

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MW- Molecular Weight

Na^{2+}/K^{+} ATPase- Sodium Potassium ATPase

NES- Nuclear Export Signal

NFAT- Calcineurin/Nuclear Factor of Activated T-Cells

NHERF2- Na^{+}/H^{+} Exchange Regulatory Factor 2

NHR - NFAT Homology Region

NLS- Nuclear Localisation Sequence

nNOS- Neural Nitric Oxide Synthase

NO- Nitric Oxide

PAD- Peripheral Arterial Disease

PAGE- Polyacrylamide Gel Electrophoresis

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PDGF-B- Platelet Derived Growth Factor-B

PDGFR – Platelet Derived Growth Factor Receptor

PDR- Proliferative Diabetic Retinopathy

PDZ- Post Synaptic Density, Drosophila Disc Large Tumour Suppressor and Zona Occludens-1

PI3K- Phosphatidylinositol-3 Kinase

PISP- PMCA Interacting Single-PDZ Domain

PKA- Protein Kinase A

PKC- Protein Kinase C

PlGF- Placental Growth Factor

PMCA- Plasma membrane calcium ATPase

PP-2B - Protein Phosphatase 2B

PVDF- Polyvinylidene Difluoride

RA- Rheumatoid Arthritis

RASSF1- Ras-Associated Factor 1

RCAN1- Regulator of Calcineurin 1

RCC- Renal Cell Carcinoma

RHR – Rel Homology Region

RIPA- Radioimmunoprecipitation Assay

RLU- Relative Light Units

RPE- Retinal Pigment Epithelium

RT PCR- Real time Polymerase Chain Reaction

SCs- Stalk Cells

SDS- Sodium Dodecyl Sulphate

SERCA - Sarcoplasmic Endoplasmic Reticulum Calcium ATPase

svVEGF- Snake venom Vascular Endothelial Growth Factor

TAD- Transactivation Domain

TBS- Tris Buffered Saline

TEMED- N,N,N',N'-Tetramethylethylenediamine

TGFβ-1- Transforming Growth Factor-B1

TM- transmembrane

TSP-1- Thrombospondin-1

Tub- Tubulin

VEGF- Vascular Endothelial Growth Factor

VEGFR- Vascular Endothelial Growth Factor Receptor

WB- Western Blot

Publications and Presentations derived from this thesis

Papers published

- **Kurusamy S**, Lopez-Maderuelo MD, Little R, Cadagan D, Savage AM, Ihugba J, Baggott RR, Rowther FB, Martinez-Martinez S, Gomez-del Arco P, Murcott C, Wang W, Oceandy D, Neyses L, Wilkinson RN, Cartwright EJ, Redondo JM & Armesilla AL (2017) SELECTIVE INHIBITION OF PLASMA MEMBRANE CALCIUM ATPASE 4 IMPROVES VEGF-MEDIATED ANGIOGENESIS. *Journal of Molecular and Cellular Cardiology,* 109, pp: 38-47.

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CHAPTER 1

Introduction

1.1 Angiogenesis

Angiogenesis is a biological process by which new blood vessels are formed from pre-existing ones (Carmeliet and Jain, 2011). It is a fundamental process that occurs both in physiological and pathological settings. In normal conditions, angiogenesis is essential for embryogenesis, organ growth, menstrual cycle, bone formation and tissue repair (Carmeliet and Jain, 2011). This physiological process is tightly regulated, involving the participation of a number of pro- and anti-angiogenic mediators (Carmeliet, 2005). An imbalance in the production of these angiogenic activators or inhibitors, results in aberrant blood vessel formation in several human diseases such as tumour growth and metastasis, diabetic retinopathy, ischaemic disorders, rheumatoid arthritis, and age-related macular degeneration (Carmeliet and Jain, 2000). As we will see in the following sections, among all the factors implicated in angiogenesis progression, vascular endothelial growth factor (VEGF) and its receptor VEGF-R have been identified as key regulators of both physiological and pathological angiogenesis (Takahashi and Shibuya, 2005).

1.1.1 Physiological angiogenesis

1.1.1a Embryonic Development

A key aspect in the development of any tissue or organ is the formation of a proper vascular network that transports oxygen and nutrients to the developing organs (Patel-Hett and D'Amore, 2011). Although this is the main function of the vascularity, it also has many other physiological tasks. Vasculogenesis is the process through which a primary vascular network is formed in the embryo (Goldie *et al.,* 2008). Vasculogenesis involves the differentiation of precursor cells called hemangioblasts into endothelial cells (Patan, 2000). Angiogenesis then extends the primitive vascular network through remodelling of the primary vessels by forming new vessels through intussusceptive angiogenesis or vascular sprouting. In the case of intussusceptive angiogenesis a pre-

existing lumen is divided into two by tissue insertion (Patan, 2000) (Figure 1.1). In sprouting, the vessel segments go through a process, involving basement membrane degradation, endothelial cell proliferation, and solid sprouts formation (Patan, 2000) (Figure 1.1). Angiogenesis begins at embryonic day E9.5 (Patel-Hett and D'Amore, 2011). The importance of this process in embryo development is well demonstrated, as attenuation of vascular formation and yolk sac development by inhibition of angiogenesis *in vivo* prevents embryo growth (Klauber *et al,* 1997). As we have mentioned before, VEGF plays an essential role in embryonic development. Lack of this pro-angiogenic factor results in embryonic lethality (Carmeliet *et al,* 1996; Ferrara *et al,* 1996) even in heterozygous animals (Hiratsuka *et al.,* 2004).

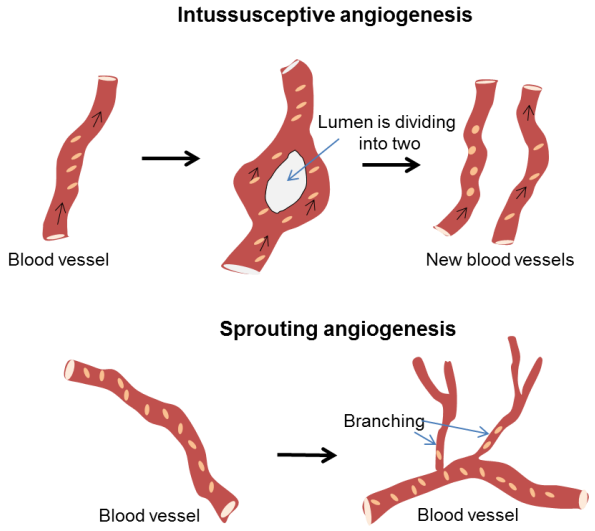


Figure 1.1 Diagrammatic description of the difference between intussusceptive and sprouting angiogenesis. In intussusceptive angiogenesis a new blood vessel is created by splitting of an existing blood vessel into two. In sprouting angiogenesis new blood vessels branch out from pre-existing ones.

1.1.b Wound healing

Wound healing is divided into four predictable phases: haemostasis (blood clotting), inflammation, proliferation (tissue growth), and extracellular matrix formation and

remodelling (maturation). Angiogenesis normally occurs during the proliferation phase (Greaves *et al*, 2013) which occurs between days 3 and 20 after wound occurrence (Bauer *et al*, 2005). The angiogenic process is essential for delivering oxygen and nutrients to the wounded area. It will also restore the capillaries that have been injured (Greaves *et al*, 2013), and helps to remove debris from the site of injury (Nissen *et al*, 1998). A significant increase in the amount of VEGF present in the area of wound is detected between 3 and 7 days after initial wound occurrence, indicating the importance of this factor in the healing process (Nissen *et al*, 1998). The VEGF present in the wound can be produced from many cell types such as keratinocytes, platelets, macrophages, neutrophils, endothelial cells, fibroblasts, or smooth muscle cells (Greaves *et al.,* 2013)

1.1.1c Menstrual Cycle

The menstrual cycle is comprised of three main phases; the menstruation phase, the proliferative phase and the secretory phase. During the menstrual cycle angiogenesis is an essential process for the restoration of the remaining layer of the endometrium and the growth of blood spiral arterioles (Demir *et al*, 2010; Maas *et al*, 2001; Shifren *et al*, 1996). Human endometrial fragments in early proliferative and late secretory phases show a significant increment in the number of vessels present in the endometrium, confirming a requirement for angiogenesis at these specific time points in the menstrual cycle (Maas *et al*, 2001). Furthermore, elevated levels of VEGF mRNA expression in human endometrium during the proliferative phase reinforce the relevance of VEGF-driven angiogenesis as an essential process for the physiology of the endometrium during the menstrual cycle (Shifren *et al*, 1996).

1.1.2 Pathological angiogenesis

Pathological angiogenesis is the result of an imbalance in the production of pro- and anti-angiogenic factors that leads to either insufficient or excessive blood vessel

formation in a wide variety of human diseases (Carmeliet, 2005). Unlike physiological angiogenesis, pathological angiogenesis results in the formation of abnormal, leaky blood vessels.

1.1.2a Pathologies associated with excessive angiogenesis

1.1.2a(i) Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a complex chronic disorder characterised by distinct autoimmune, inflammatory and fibrovascular components which lead to synovial proliferation and joint destruction (Stupack *et al*., 1999). Sone *et al*, 2001 reported that the severity of the disease positively correlates with increments in the amount of VEGF in the synovium (Sone *et al*, 2001). During RA the synovium undergoes inflammation and increments in mass, generating a hypoxic environment that triggers upregulation of *VEGF* gene expression and the subsequent increase in angiogenesis (Paleolog, 2002). The newly formed blood vessels not only transport oxygen and nutrients to support the proliferation of synovial cells, but also localise inflammatory cells in the area leading to inflammation of the synovium (Marrelli *et al*, 2011).

1.1.2a(ii) Psoriasis

Psoriasis is a common chronic skin disorder characterized by epidermal hyperplasia, cutaneous inflammation and increased angiogenesis (Singh *et al.,* 2013). In particular, microvessels of the papillary dermis are elongated and dilated, resulting in increased permeability (Detmar *et al.,* 1994). VEGF and VEGF receptors VEGFR-1 and 2 have been identified as major epidermis-derived vessel-specific growth factors strongly up-regulated in the hyperplastic epidermis (Li *et al.,* 2014). This fact highlights the link between augmented angiogenesis and the increased demand of nutrients and oxygen at hyperplastic sites that characterise psoriasis lesions (Detmar *et al.,* 1994).

1.1.2a(iii) Retinopathies

Several studies have suggested a role for angiogenesis in retinopathy (Aiello *et al.,* 1994; Adamis *et al.,* 1994). According to these studies, elevated levels of VEGF in the vitreous and aqueous fluid in the retina of retinopathy patients lead to abnormal formation of new and highly permeable blood vessels in the eye of the patient (Cheung *et al.,* 2010).

Choroidal neovascularisation in wet age-related macular degeneration (AMD) is a severe ocular complication associated with abnormal vessel growth in the retina (Kourlas and Abrams, 2007; Zhou and Wang, 2006) that causes vessel leaking and haemorrhage, and results in retinal pigment epithelium detachment (Kourlas and Abrams, 2007). AMD is the most common cause of irreversible sight loss in elderly patients (Zhou and Wang, 2006). Cheung *et al*, 2010 showed that the expression of VEGF is elevated in retinal endothelial cells and pericytes of diabetic retinopathy patients due to the presence of a hypoxic environment within the eye (Cheung *et al*, 2010; Adamis et al, 1994). It is thought that elevated levels of glucose in the blood of the patient cause apoptosis of endothelial cells in the choroid leading to vessel rarefaction and generating a hypoxic micro-environment in the eye that, ironically, promotes the formation of new, leaky blood vessels (Shin *et al.,* 2014). Haemorrhage and fluid leaking from this abnormal vasculature lead to an increase in ocular fluid that elevates intraocular pressure and severely damages the patient's eye (Kourlas and Abrams, 2007).

1.1.2a(iv) Endometriosis

Endometriosis is a chronic inflammatory disease characterized by the presence and growth of endometrial tissue outside the uterine cavity (Hull *et al*, 2003). Angiogenesis plays an essential role in the development of a vascular supply and growth of endometric lesions (Hull *et al*, 2003). Increased level of VEGF expression has been

demonstrated in the peritoneal fluid of women suffering endometriosis and this correlates with the severity of the disease (Shifren *et al*, 1996).

1.1.2a(v) Tumour Angiogenesis

When a tumour reaches a size of 1-2mm, it requires high levels of nutrients and oxygen so the tumoral cells can live and keep growing (Bergers and Benjamin, 2003). This supply of nutrients along with oxygen is facilitated by angiogenesis (Papetti and Herman, 2002). Angiogenesis is also reported to be one of the key reasons for the metastatic spread of tumours (Jain, 2005). Tumour vessels are reported to be leaky, disorderly, dilated and often to have dead ends resulting in reduced blood flow to the tumour (Bergers and Benjamin, 2003). In tumours, pericytes also appear to be more loosely attached to the vasculature leading to incomplete vessel maturation (Bergers and Benjamin, 2003). Tumours that are actively proliferating are reported to introduce additional stress on the vasculature, leading to constricted blood flow into tumours (Jain, 2005). Elevated levels of VEGF are linked to increase in microvessel density and decrease in the survival rate of patients (Toi *et al*, 1994) indicating that as in other aspects of physiological and pathological angiogenesis, the axis VEGF-VEGFR plays also a pivotal role in the progression of angiogenesis in tumours (Chen *et al*., 2012).

1.1.2b Pathologies associated with insufficient angiogenesis

1.1.2b(i) Ischaemic heart disease

Ischaemic heart disease is a condition that affects the supply of blood to the heart. The coronary arteries become narrowed or blocked due to gradual deposition of fatty material on their walls. This condition is known as atherosclerosis. Progression of the disease is associated with the growth and/or episodic rupture of atherosclerosis lesions leading to narrowing or occlusion of major coronary arteries and resulting in insufficient coronary perfusion to match the myocardial oxygen demand (Koerselman *et al*., 2003). It is well established that cardiac ischaemia activates vascular regrowth responses in

the heart to protect the myocardial tissue against the ischaemic condition by sprouting existing capillaries (angiogenesis) and by developing collateral vessels (arteriogenesis) in the occluded coronary artery that bypass the area of stenosis or occlusion (Seiler, 2010). Unfortunately, patients with similar degrees of coronary stenosis show a marked variability in the development of new capillary and collateral artery vessels, and reperfusion of the ischaemic heart is not efficient in many cases (Hansen *et al.,* 1989). Moreover, formation of new blood vessels is significantly impaired in patients with type 2 diabetes, metabolic syndrome, and severe atherosclerosis (Rocic *et al*., 2012; Turgut *et al*., 2009).

1.1.2b(ii) Peripheral arterial disease

Peripheral arterial disease (PAD) is mainly caused by occlusive atherosclerosis in a vascular bed other than the heart or the brain. PAD most commonly affects the lower extremity, but other arteries may also be involved (Brostow *et al*., 2012). Critical limb ischaemia (CLI) is considered the most-severe clinical manifestation of PAD (Annex, 2013). The arterial narrowing or obstruction of arteries in the lower limbs impairs blood flow to the lower extremities consequently causing limb pain, ulcers, gangrene and in severe cases amputation (below the knee or higher) (Annex, 2013).

1.1.2b(iii) Stroke

Stroke is a devastating disease caused by occlusion of a cerebral artery followed by disturbances in blood supply through microvessels to the brain tissue leading to severe brain damage (Seto *et al*., 2016). Therapeutic approaches for patients with acute ischaemic stroke focus on neuroprotection, thrombolysis and surgical clot removal. Recent studies have shown that new vessel formation after ischaemic stroke not only supplies blood to the ischaemic tissue, but also promotes neurogenesis and improves neurological functions in both animal models and patients (Seto *et al*., 2016). Issa *et al.,* 1999 reported that VEGF and its receptors were up-regulated in human brain tissue

after ischaemic stroke (Issa *et al.,* 1999). Several studies in animal models proposed that an intra-cerebroventricular injection of VEGF reduced neurological deficits after focal cerebral ischaemia (Issa *et al*., 1999). Unfortunately, VEGF treatment was reported to lead to blood-brain barrier (BBB) leakage, brain edema, vasodilation and aberrant systemic hemodynamics (Issa *et al*., 1999).

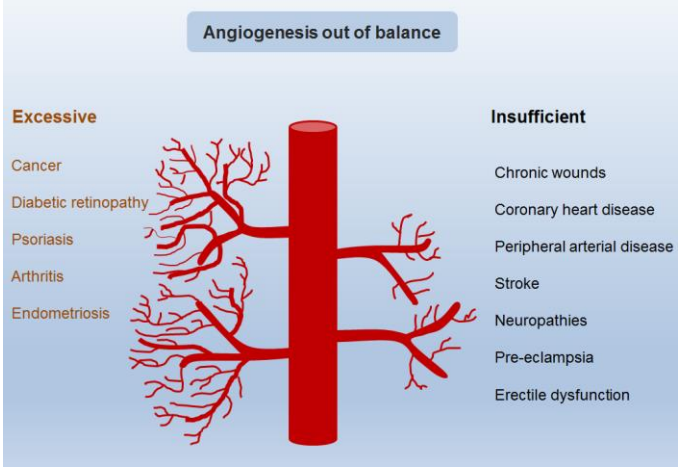


Figure 1.2 Schematic diagram describing major pathological disorders in humans associated with angiogenesis deregulation.

1.1.3 Cellular events required for successful angiogenesis

Angiogenesis is a complex multistep process involving extensive interplay between cells, soluble factors, and extracellular matrix components (ECM) (Ribatti *et al*., 2012). In a quiescent state, endothelial cells form a monolayer of inactivated cells that lines the luminal surface of stable vessel tubes (Mazzone *et al*., 2009). In response to pro-angiogenic factors, endothelial cells loosen their cell-cell junction contacts and pericytes and/or smooth muscle cells are removed from the existing vessel (Figure 1.3). During this process, proteases such as plasmin and matrix metalloproteinases (MMPs) are activated promoting degradation of the surrounding basement membrane, and allowing endothelial cells to invade the surrounding tissue and respond to environmental factors (Carmeliet and Jain, 2011; Adams and Alitalo, 2007). Only a small portion of endothelial cells (‘‘tip cells’’) sense the angiogenic stimulus and will lead the newly sprouting blood

vessel (Figure 1.3). Endothelial tip cells (ETCs) continue sprouting further by filopodial extension, in a process guided by pro-angiogenic growth factors and involving semaphorins and ephrins (De Smet *et al.,* 2009; Gerhardt *et al*., 2003). A variety of pro-angiogenic inducers; such as VEGF, basic fibroblast growth factor (bFGF), angiopoietins, cytokines, chemokines, and others induce proliferation of endothelial cells (Ucuzian *et al.,* 2010). ETCs are followed by endothelial 'stalk cells' (SCs) which support the elongation of sprouting vessels, generate the trunk of the new vessel, and connect the sprouting with the parental vessel (Iruela-Arispe *et al.,* 2009; Kamei *et al.,* 2006). Upon contact with other vessel walls, ETCs lose their motile behaviour and fuse with the tip cell of another vessel by a process called anastomosis (Figure 1.3). The mechanisms underlying the process of anastomosis are not well understood, but it is believed that vessel fusion is influenced by a certain type of macrophages along with myeloid cells during the anastomotic process (Fantin *et al.,* 2010). Even though nascent vasculature has been generated in this process they are still termed as 'immature' as they require maturation to acquire full functionality (Liekens *et al*., 2001). The final stage of angiogenesis requires recruitment of pericytes and smooth muscle cells to the newly formed vessel, which is induced by growth factors such as platelet derived growth factor-B (PDGF-B) and transforming growth factor-β1 (TGFβ-1). Additionally, deposition of the endothelial basement membrane and cell–cell junction stability suppress endothelial proliferation, migration and vascular permeability, and re-establishes a quiescent stage (Herbert and Stainier, 2011) (Figure 1.3).

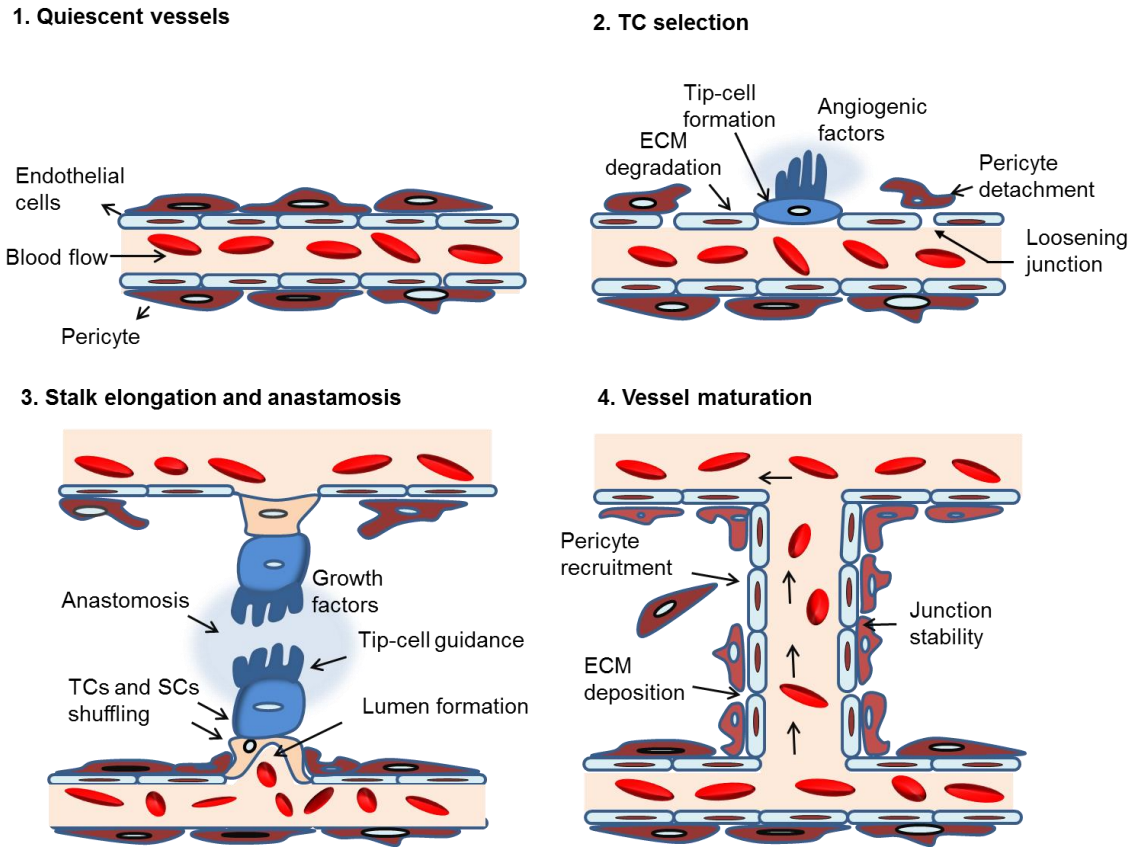


Figure 1.3 Diagrammatic description of the steps involved in angiogenesis. (adapted from Carmeliet and Jain, 2011; Herbert and Stainier, 2011). ECM, extracellular matrix; TCs, tip cells; SCs, stalk cells.

1.1.4 Molecular regulation of angiogenesis

As we have mentioned earlier, regulation of angiogenesis is tightly controlled by multiple pro- and anti-angiogenic molecules. Most angiogenic inducers are proteins, mainly growth factors which stimulate endothelial cells to proliferate and migrate towards the stimuli. Up to 24 inducers of angiogenesis have been identified so far. The best studied of these pro-angiogenic factors are mentioned in table 1.1

On the other hand, angiogenesis is down-regulated by a variety of inhibitors including angiostatin, endostatin, thrombospondin-1 (TSP-1) and others (Table 1.1). In this sense, Eriksson *et al,* showed that both angiostatin and endostatin inhibit endothelial cell migration in the presence of the angiogenic activators VEGF and FGF (Eriksson *et al.,*

2003). Similarly, the anti-angiogenic effects of TSP-1 have been clearly defined in studies on angiogenesis in the tumour microenvironment (Good *et al.,* 1990).

Table 1.1 Regulators of angiogenesis

Pro-angiogenic factors (Activators)	Anti-angiogenic factors (Inhibitors)
Angiopoietin-1 and 2	Angiostatin
Fibroblast growth factor (bFGF)	Anti-thrombin III fragment
Cyclooxygenease-2 (COX-2)	Interferon (INF-α, -β, -γ)
Insulin like growth factor (IGF)	Platelet factor-4
Metalloproteinase (MMP's)	Tissue inhibitors of MMP (TIMPs)
Nitric oxide synthase	Thrombospondin-1 (TSP-1)
Vascular endothelial (VE)-cadherin	Endostatin
Plasminogen activator	Vasostatin
Vascular Endothelial Growth Factor (VEGF. VEGF-B, VEGF-C, VEGF-F)	
Hypoxia-inducible factor-1α (HIF-1α)	
Interleukin-8 (IL-8)	
Platelet-derived growth factor (PDGF)	
Tumor necrosis factor-α (TNF-α)	

Among all angiogenesis effectors, VEGF has emerged as a critical regulator of both pathological and physiological angiogenesis (Takahashi and Shibuya, 2005). Given the relevance of VEGF-dependent angiogenic stimulation in the work presented in this thesis, I will describe in detail the features and mechanism of action of this growth factor.

1.1.5 The vascular endothelial growth factor protein family

VEGF belongs to a protein family called the vascular endothelial growth factor (VEGF) family, that comprises seven members; VEGF-A, VEGF-B, VEGF-C VEGF-D, VEGF-E, svVEGF (snake venom VEGF) and PLGF (placental growth factor) (Takahashi and Shibuya, 2005). These proteins bind to three structurally different receptor tyrosine

kinases known as VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) (Holmes *et al.,* 2007). The molecular and biological function of each ligand and its receptors are thoroughly characterized. Each of these ligands has distinct tissue distribution, interaction with different receptor tyrosine kinases and diverse effects on the angiogenic process (Takahashi and Shibuya, 2005). I will focus on VEGF-A in this thesis, as it acts on endothelial cells and is implicated in both physiological and pathological angiogenesis.

1.1.5a VEGF-A

VEGF-A is the most potent stimulator of angiogenesis. It is produced by a wide variety of cells, organs and tissues including tumour cells, the corpus luteum, the heart, liver, stomach mucosa, adrenal cortex, cardiac myocytes, renal glomeruli, epithelial cells of the lung and macrophages (Berse *et al.,* 1992). The importance of VEGF-A in vascular function was highlighted by the fact that inactivation of one or both alleles results in embryonic lethality at approximately 10.5 days, due to lack of vascular structure formation (Cameliet *et al.,* 1996). Another study demonstrated that heterozygous knock-out of VEGF-A results in embryonic lethality between days 11 and 12, with impairment of angiogenesis and blood-island formation (Ferrara *et al*., 1996).

In humans, the *VEGF-A* gene is located on chromosome 6p21.3 (Vincenti *et al*., 1996). *VEGF-A* is composed of seven introns and eight exons (Figure 1.4).

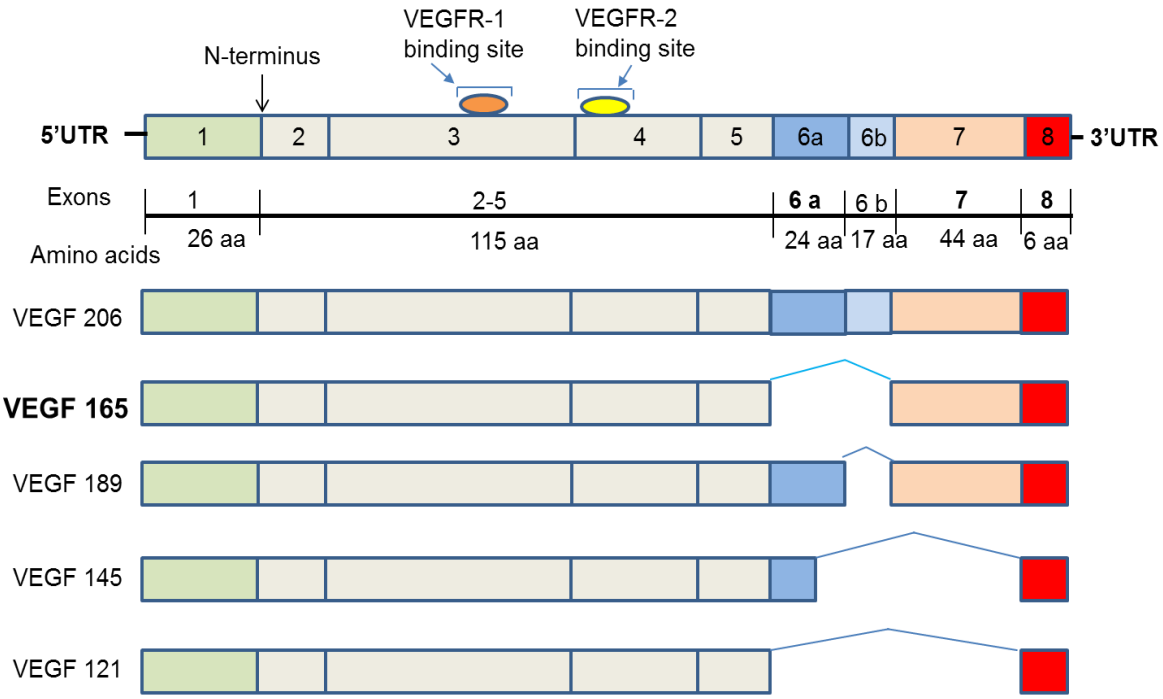


Figure 1.4 Schematic diagram of major human VEGF-A mRNA splice variants (adapted from Holmes *et al.,* 2007). *VEGF-A* comprises seven introns and eight exons that encode different structural isoforms. Alternative splicing of this RNA generates several different isoforms that vary in amino acid number; VEGF-A121, VEGF-A145, VEGF-A148, VEGF-A162, VEGF-A165, VEGF-A183, VEGF-A189, VEGF-A206. In humans, VEGF-A165 is the most predominant form, and the major stimulator of angiogenesis.

The VEGF-A protein exists in a number of different isoforms that result from alternative splicing of a single precursor RNA (Figure 1.4). In humans, nine VEGF-A splice variants have been detected: VEGF-A121, VEGF-A145, VEGF-A148, VEGF-A162, VEGF-A165, VEGF-A183, VEGF-A189, VEGF-A206 and also VEGF-A165b (Holmes et al., 2007). Although VEGF-A121, VEGF-A183 and VEGF-A189 are expressed in various tissues, VEGF-A165 is the most abundantly expressed isoform and has been identified as the main regulator of physiological and pathological angiogenesis (Robinson and Stringer, 2001; Takahashi and Shibuya, 2005). Consistent with a role in pathological angiogenesis, VEGF-A165 is strongly expressed by tumoral cells, and its expression correlates with tumor progression (Ferrara N, 2004). Likewise, we have also mentioned in previous sections that elevated levels of VEGF have been detected in the vitreous

and aqueous fluids of patients with diabetic retinopathy (Aiello et al., 1994; Adamis et al., 1994).

1.1.5b VEGF Receptors

The biological effects of VEGF are mediated through binding to specific cell receptors (VEGFR) which are predominantly found in endothelial cells, although they are also expressed in other cell types. VEGFRs actively participate in the regulation of angiogenesis, vasculogenesis and lymphangiogenesis (Lohela *et al.,* 2009).

There are three VEGF receptors (VEGFR-1, -2, and -3) with different ligand-binding properties and tyrosine kinase activity (Takahashi and Shibuya, 2005; Ferrara *et al.,* 2003). Structurally, VEGFRs have an extracellular portion consisting of seven immunoglobulin-like domains, a single transmembrane spanning region and an inner cytoplasmic portion containing a split tyrosine-kinase domain (Figure 1.5) (Takahashi and Shibuya, 2005; Ferrara *et al.,* 2003).

VEGFRs function similarly to other tyrosine kinase receptors such as epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR), and platelet derived growth factor receptor (PDGFR) (Olsson *et al.,* 2006). Ligand interaction with its corresponding receptor induces receptor dimerization that creates a docking site for signalling and, consequently, activation of the tyrosine kinase domain leading to receptor auto-phosphorylation (Stuttfeld and Ballmer-Hofer, 2009; Olsson *et al.,* 2006).

VEGFR-1 and VEGFR-2 are expressed in endothelial cells (Olsson *et al*., 2006). VEGFR-1 is also expressed in monocytes, macrophages and haematopoietic stem cells, playing an important role in the migration of these cellular types (Yao *et al.,* 2011). VEGFR-3 is expressed in the lymphatic endothelium and plays a fundamental role in lymphangiogenesis (Olsson *et al.,* 2006) (Figure 1.5)

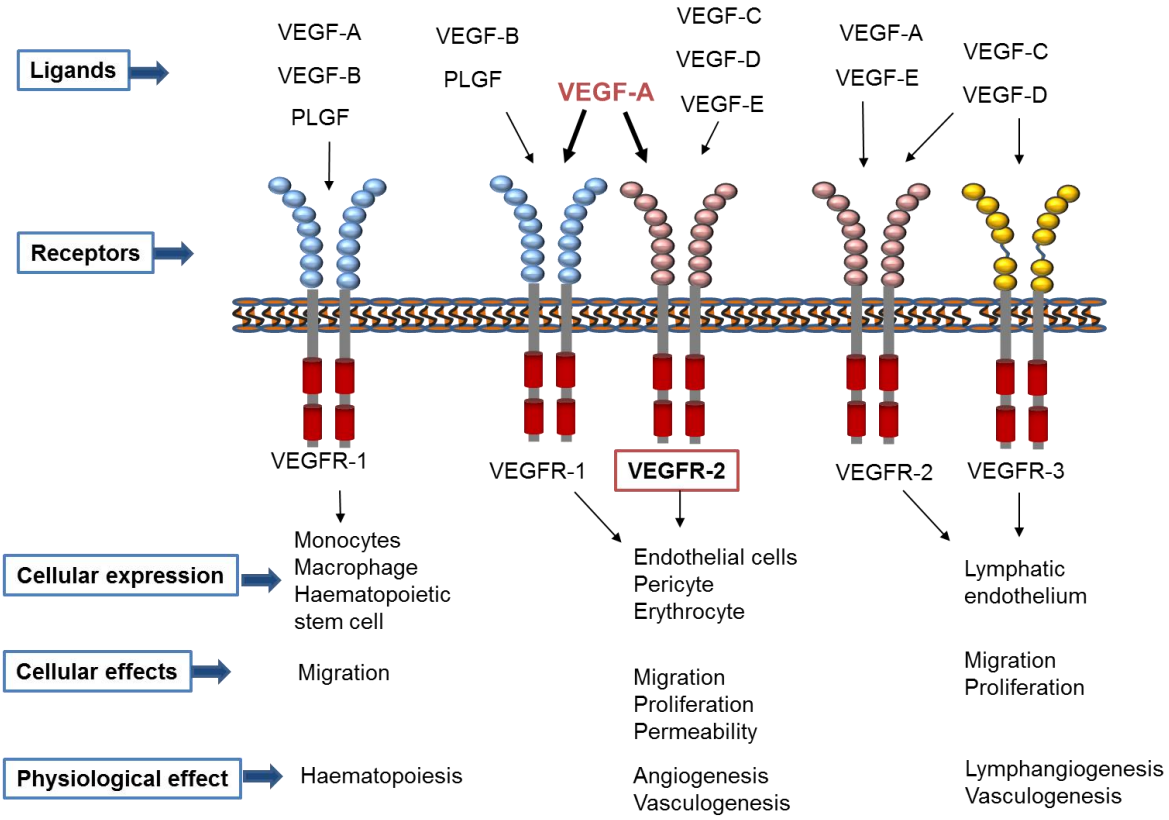


Figure 1.5 VEGF receptor binding properties, expression and downstream functional effects (adapted from Holmes *et al*., 2007).

1.1.5b(i) VEGFR-1

VEGFR-1 is a 180-185 kDa glycoprotein that binds VEGF-A, VEGF-B and PIGF (Figure 1.5) (Takahashi and Shibuya, 2005). The role of VEGFR-1 has been studied using knockout mouse models. Deletion of the *VEGFR-1* gene in the mouse embryo is lethal at embryonic day E9.0 due to an increased number of vessels in the vasculature (Fong *et al.,* 1995). Recent studies have shown that VEGFR-1 does not mediate VEGF-driven angiogenesis in endothelial cells. Instead, VEGFR-1 acts as a decoy receptor controlling the binding and signalling of VEGF to VEGFR-2 (Ferrara *et al.,* 2003).

1.1.5b(ii) VEGFR-2

VEGFR-2, also known as kinase-insert domain receptor (KDR) and foetal liver kinase-1 (Flk-1) is a 210-230 kDa glycoprotein that plays a critical role in endothelial survival,

proliferation, migration, and vascular development upon stimulation by VEGF-A (Figure 1.5) (Terman *et al.,* 1991; Takahashi and Shibuya, 2005).

The *VEGFR-2* gene is located on chromosome 4q11q12, in close proximity to the locus of another two receptors, PDGFR-α and C-KIT (Shibuya, 2002). Studies in *VEGFR-2*$^{-/-}$ mice have revealed that embryos deficient in VEGFR-2 die between days E8.5 and E9.5 due to lack of blood vessel organisation in the yolk sac and blood island formation, suggesting that VEGFR-2 is essential for the vascularisation of the embryo in early development (Shalaby *et al.,* 1995). VEGF-A, -C, -D, and -E can bind to VEGFR-2 triggering different physiological effects (Holmes *et al.,* 2007). Binding of VEGF-A to VEGFR-2 activates numerous intracellular signalling pathways that trigger specific cellular processes involved in angiogenesis. Therefore the axis VEGF-A/VEGFR-2 has emerged as a critical regulator of both physiological and pathological blood vessel formation (Takahashi and Shibuya, 2005).

1.1.6 Anti-angiogenesis therapies

1.1.6a Inhibitors of the interaction VEGF-VEGF receptor

1.1.6a(i) Bevacizumab (Avastin®)

Bevacizumab has been the first anti-angiogenic drug used therapeutically (Shih and Lindley, 2006). It is a humanized anti-angiogenic monoclonal antibody and it was approved by the Food and Drug Administration (FDA) for treating metastatic and colorectal cancer in 2004. It has a long half-life of 17-21 days (Ferrara *et al.,* 2004). It binds to VEGF with high specificity, thereby blocking the interaction between VEGF and its receptor and, subsequently, VEGF-mediated angiogenic signalling pathways (Shih and Lindley, 2006). This drug binds to all isoforms of VEGF. Bevacizumab has some possible side effects including impaired wound healing, proteinuria and severe hypertension (Shih and Lindley, 2006; Hurwitz *et al*, 2004). Kamba and McDonald, 2007 reported that hypertension is thought to be the result of attenuation in nitric oxide

production by the endothelial nitric oxide synthase (eNOS) due to reduced eNOS activation as a consequence of VEGF blockage (Kamba and McDonald, 2007). The severe side effects associated with this therapy are due to long half-life of bevacizumab (Pieramici and Rabena, 2008). Bevacizumab has been approved in combination with 5-fluorouracil-based chemotherapy for treatment of colorectal carcinoma. Use of bevacizumab in combination with 5-fluorouracil results in a reduction in blood volume, interstitial fluid pressure, and micro-vascular density in tumours (Willet *et al*, 2004).

1.1.6(ii) Pegaptanib (Macugen®, EYE001, NX1838)

Pegaptanib is an anti-angiogenic medicine for the treatment of neovascular wet age-related macular degeneration (AMD). Pegaptanib, was approved in 2004 by the FDA for the treatment of wet AMD (Zhou and Wang., 2006). It is a single stranded nucleic acid aptamer containing 28 bases (Fraunfelder, 2006) that binds specifically and inhibits the activity of VEGF-A165 by blocking its interaction with VEGFR-2 (Pieramici and Rabena, 2008). Administration of pegaptanib has been reported to significantly reduce vessel permeability, leakage, and neovascularisation in the retina of patients suffering from AMD (Gragoudas *et al*, 2004). The side effects associated to this drug are highly reduced and manageable (Zhou and Wang, 2006). In fact, most of adverse effects are related with the injection procedure rather than the drug itself (Gragoudas *et al*, 2004). Pegaptanib is also being considered for anti-angiogenic cancer treatment given the success obtained in the treatment of neovascular-related ocular disease.

1.1.6a(iii) Ranibizumab (Lucentis®)

Ranibizumab is a humanised monoclonal antibody fragment (Fab) from bevacizumab that was FDA approved in 2006. It binds to all isoforms of VEGF-A, inhibiting their activity by blocking the VEGF/VEGFR interaction and the initiation of intracellular signalling (Kourlas and Abrams, 2007). It has an approximately 20 times higher binding affinity for VEGF than bevacizumab (Pieramici and Rabena, 2008). Additionally, it can

penetrate better in tissues due to its small size compared to bevacizumab (Pieramici and Rabena, 2008). Ranibizumab treatment in patients with neovascular AMD reduced loss of visual acuity compared to sham-treated control patients. Few adverse effects were observed with the administration of this treatment, most severe being endophthamitis (Rosenfeld *et al*, 2006).

1.1.6a(iv) VEGF-Trap

VEGF-Trap is another inhibitor of VEGF that binds and inhibits the function of VEGF by preventing its interaction with VEGFR. It is generated using the second and third Ig domains of VEGFR-1 and VEGFR-2 respectively (which are the regions of the receptor involved in VEGF binding in each case). These regions are combined with a human IgG Fc fragment and the resulting molecule has the highest affinity for VEGF compared to other drugs (Chang *et al*, 2012; Holash *et al*, 2002). Additionally, VEGF-Trap is smaller in size than other inhibitors and therefore penetrates better in tissues (Pieramici and Rabena, 2008). It binds to all isoforms of VEGF-A, and additionally placental growth factor (PlGF-1 and -2) (Chang *et al*, 2012). Treatment of patients presenting neovascular AMD using VEGF-Trap resulted in a reduction in lesion size and choroidal neovascularisation and significantly improved visual acuity. Adverse effects are mainly conjunctival hemorrhage associated with the injection procedure. Few systemic side effects were observed such as bronchitis and infection of the upper respiratory and urinary tract (Heier *et al*, 2011).

1.1.6b VEGFR Inhibitors

VEGFR inhibitors are mainly monoclonal antibodies and receptor tyrosine kinase inhibitors (Casanovas *et al*, 2005). Monoclonal antibodies bind to the surface of VEGFR and impair binding of VEGF. Receptor tyrosine kinase inhibitors (such as small molecule inhibitors) bind directly to the ATP binding site in the cytosolic region of the receptor, and prevent both ATP binding and activation of signalling pathways (Gotink

and Verheul, 2010). Problems associated to treatments based on drugs inhibiting receptor tyrosine kinases are the low specificity given the huge number of receptors with tyrosine kinase activity in the cell.

1.1.6b(i) Sunitinib (Sutent®, SU11248)

Sunitinib, approved in 2006 by the FDA (Faivre *et al*, 2007), is a small molecule inhibitor that can bind directly to the ATP binding site of VEGFR, preventing both ATP binding and receptor activation (Gotink and Verheul, 2010). It inhibits cellular signalling by targeting multiple receptor tyrosine kinases. These include VEGFR-1, 2 and 3 and the platelet-derived growth factor receptor (PDGFR) (Faivre *et al*, 2007). Sunitinib treatment of patients and animal models with several kinds of tumour resulted in a significant reduction in tumour growth and microvessel density (Mendel *et al*, 2003; Motzer *et al*, 2006). Side effects were observed such as diarrhoea, vomiting, fatigue, hypertension and hand-foot syndrome (delicate skin on the hands and feet that can blister and peel in severe cases) (Faivre *et al*, 2007). As sunitinib has a short-life it is suggested the recovery rate of adverse effects will be quicker (Kamba and McDonald, 2007).

1.1.6b(ii) Sorafenib (Nexavar®, Bay 43-9006)

Sorafenib was FDA approved in 2005 for the treatment of renal cell carcinoma (RCC) (Wilhelm *et al*, 2006). It is also a small molecule inhibitor that targets the intracellular region of VEGFR. This small molecule can pass through the membrane and bind to cytosolic regions of the receptor at a site next to the ATP binding site, preventing receptor interaction with ATP and signal transduction activation (Gotink and Verheul, 2010; Wilhelm *et al*, 2006). Administration of sorafenib has been reported to reduce tumour growth and microvessel density in animal models and clinical trials of human patients (Wilhelm *et al*, 2004; Ratain *et al*, 2006). Unfortunately, this molecule is associated with some side effects including hypertension, fatigue, hand-foot syndrome and diarrhoea which could be reduced by dose reduction (Ratain *et al*, 2006).

1.1.6b(iii) IMC-1121b (Ramucirumab)

IMC-1121b is a monoclonal antibody that targets VEGFR. It binds to VEGFR-2 and it works as a receptor antagonist, blocking the binding of all isoforms of VEGF to this receptor (Spratlin *et al*, 2010). Administration of ramucirumab in patients with advanced solid malignancies resulted in reduced perfusion and vascularity of tumours. The common side effects of ramucirumab include hypertension, proteinuria, abdominal pain, headaches, deep vein thrombosis (DVT) and vomiting (Spratlin *et al*, 2010).

1.1.7 Pro-angiogenesis therapies

Although interventional endovascular procedure and coronary artery bypass surgery are effective treatments to restore the blood flow to the ischaemic heart, some patients are unsuitable for surgical revascularization (Sachs *et al*., 2011). Therapeutic strategies to promote the formation of new blood vessels in the ischaemic organ (referred to as therapeutic angiogenesis) represent a promising alternative to treat these patients (Zachary and Morgan, 2011; Cooke and Losordo, 2015). The aim of therapeutic angiogenesis is to use angiogenic factors to induce the formation of a collateral blood supply, effectively bypassing an occluded diseased blood vessel in patients with coronary or peripheral artery disease, and thereby revascularising the ischaemic tissue (Deveza *et al*., 2012). To exploit the powerful angiogenic potential of VEGF, gene-, and protein-based therapeutic approaches aimed to deliver exogenous VEGF to ischaemic tissues have been developed in the last decades (Zachary and Morgan, 2011). A large body of data obtained from preclinical studies carried out in animal models of myocardial and limb ischaemic disease demonstrated that VEGF-based pro-angiogenic therapies result in successful reperfusion of the ischaemic organ (Ylä-Herttuala, 2013).

Recent studies showed that intracoronary and intravenous administration of recombinant VEGF-A165 (rhVEGF) to patients with coronary artery disease improved myocardial perfusion and collateralisation (Hendry *et al*., 2001; Hendel *et al*., 2000). A

subsequent phase II trial “VEGF in Ischaemia for Vascular Angiogenesis (VIVA)” has indicated that intravenous delivery of VEGF is safe but does not translate into a significant improvement of patients after 60 days of treatment, only a high dose of VEGF resulted in significant improvement at 120 days in angina patients.

The Isner group first established an alternative way (gene therapy) of delivering therapeutic VEGF to achieve a more sustained therapeutic effect. The authors reported that delivery of a cDNA plasmid encoding VEGF-A165 (phVEGF165) improved myocardial perfusion after 3 months (Symes *et al.,*1999; Vale *et al.,* 2000; Losordo *et al.,* 1999). Unfortunately, disappointing results were obtained later with other phase II trials (Rissanen and Ylä-Herttuala, 2007). Intramuscular injection of phVEGF-165 to patients with peripheral artery disease and critical limb ischaemia also produced negative results (Kusumanto *et al.,* 2006).

Adenovirus-mediated delivery of different VEGF isoforms (VEGF121 and VEGF165) has also been assayed as a treatment for cardiovascular ischaemic disease. A large body of phase I trials showed that this method of VEGF delivery is safe and significantly improved clinical benefits, either when given alone or as an adjutant to coronary artery bypass grafting (Rosengart *et al.,* 1999). However, in phase II trials, administration of adenovirus encoding VEGF-121 by percutaneous injection has failed to provide evidence of clinical benefits (Zachary and Morgan, 2011). Similarly, delivery of Ad-VEGF121 to patients suffering from peripheral artery disease did not promote any improvement (Rajagopalan *et al*., 2003).

It is clear that trials of therapeutic angiogenesis on human cardiovascular ischaemic diseases have only shown limited benefits for patients thus far (Gupta *et al.,* 2009). The reasons behind lack of clinical success for therapeutic angiogenesis are complex, but failure to restore efficient VEGF activity in the ischaemic organ remains a major problem in current approaches (Ylä-Herttuala, 2013; Gupta *et al.,* 2009; Zachary and Morgan*,*

2011). There is an urgent need to identify key regulators of pro-angiogenic signal transduction pathways activated by VEGF, to design more efficient interventions that enhance VEGF-driven angiogenesis in ischaemic organs. In this sense, the detail characterisation of the intracellular signalling pathways activated by VEGF binding to VEGFR-2 in endothelial cells is receiving a lot of attention.

1.1.8 VEGFR-2 signalling pathways in angiogenesis

Signalling pathways activated by binding of VEGF-A to VEGFR-2 include the Extracellular Signal-Regulated Kinase (Erk) pathway, the p38 Mitogen-Activated Protein Kinase (MAPK) pathway, activation of phospholipase C, the phosphatidylinositol 3-kinase/Akt pathway and the calcineurin/Nuclear Factor of Activated T-cells (NFAT) pathway.

1.1.8a Extracellular signal-regulated kinase (Erk) pathway

The Erk signalling cascade is considered a central MAPK pathway that plays an important role in the regulation of endothelial cell proliferation, differentiation, and cell cycle (Yoon and Seger, 2006). The Erk pathway is activated by sequential phosphorylation of protein kinases in different points of the cascade (Shaul and Seger, 2007). Binding of VEGF to VEGFR-2 triggers activation of the signalling protein Ras. Activated Ras phosphorylates the MAP kinase kinase kinase (MAPKKK) Raf that becomes activated by this phosphorylation. Activated Raf phosphorylates the MAP kinase kinase (MAPKK) MEK1/2 which consequently phosphorylates the MAP kinase (MAPK) Erk1/2. Specific inhibitors that block the phosphorylation of Erk1/2 significantly attenuate VEGF-induced endothelial cell migration and proliferation, confirming the involvement of this pathway in these angiogenic processes (Meloche and Pouyssegur, 2007; Dellinger and Brekken, 2011).

1.1.8b P38 Mitogen Activated Protein Kinase

The p38 MAPK family consists of four isoforms; p38-α, -β, -γ, and -δ. This protein becomes phosphorylated (and therefore activated) by an upstream MAPKK called MEK3/6. Pharmacologic inhibition of p38 attenuates VEGF-stimulated cell migration (McMullen *et al.,* 2005; Rousseau *et al.,* 1997).

1.1.8c Phosphatidylinositol-3 Kinase (PI3K)

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that play an important role in various cellular functions including cell proliferation, adhesion, migration, survival and angiogenesis (Sasore and Kennedy, 2014 and Karar and Maity, 2011). The PI3K is mainly activated by tyrosine kinase receptor and G-protein-coupled receptors. Given the huge variety of physiological roles of the PI3K we will focus on this section on describing its role in angiogenesis as this is the main subject of this thesis. Several works have demonstrated that VEGF induced activation of PI3K signalling in endothelial cells is essential for the progression of angiogenesis (Tsuji-Tamura and Ogawa, 2016). The PI3K signalling pathway plays an important role in the formation of normal blood vessel during embryonic development (Hamada, 2005). Activation of PI3K/Akt signalling translates into increased expression of VEGF in tumor cells further activating endothelial angiogenic signalling (Cheng *et al.,* 2017). Furthermore, PI3K/Akt modulates the expression of other angiogenic factors including Nitric Oxide (NO) and angiopoietin. Hence, the PI3K pathway is important in both physiological and pathological blood vessel formation.

The PI3K family is composed of three classes of PI3K; class I, class II, class III. This classification is mainly based on their domain structures and lipid surface preferences. Class I PI3K composed of 4 catalytic isoforms; p110α (PIK3CA), p110β (PIK3CB), p110γ (PIK3CB), and p110δ (PIk3CD) (Karar and Maity, 2011and Graupera and

Potente, 2013). Although these four isoforms are expressed in endothelial cells, only p110α is required for vessel sprouting (Soler *et al.,* 2015). Deletion of kinase p110α catalytic subunit of PI3K in mice lead to vascular defect during the development stage (Karar and Maity, 2011). The involvement of class II and class III PI3K in angiogenesis is not understood at present.

1.1.8d Protein Kinase C (PKC)

PKC contains at least 10 isoenzymes grouped into three classes; conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC). PKC signalling enhances angiogenic activity in endothelial cells induced by VEGF stimulation (Xu *et al.,* 2008; Harhaj *et al.,* 2006).

1.1.8e Calcineurin/NFAT pathway

Several works have demonstrated that VEGF-induced activation of the calcineurin/NFAT pathway in endothelial cells is an essential step in the progression of VEGF-driven angiogenesis (Armesilla *et al.*, 1999; Hernandez *et al.,* 2001; Schabbauer *et al*., 2007; Ryeom *et al.*, 2008).

Given the relevance of VEGF-induced activation of the calcineurin/NFAT pathway for the topic of this thesis, I will describe in detail the molecular features of this pathway in the following sections.

1.2 The calcineurin/NFAT pathway

1.2.1 Calcineurin

Calcineurin is a calcium and calmodulin-dependent serine/threonine phosphatase of approximately 80 kDa. It is also known as protein phosphatase 2B (PP-2B) (Rusnak and Mertz, 2000). Originally identified in the brain, calcineurin was later shown to have a role in diverse biological processes such as regulation of the immune response (Snyder *et al.*, 1998), apoptosis (Klee *et al*., 1998) and angiogenesis (Hernandez *et al.,* 2001).

Additionally, it plays an important role in cardiac hypertrophy (Molkentin, 2004) and central nervous system functions (CNS) (Winder and Sweatt, 2001).

Calcineurin is a heterodimeric protein composed of two subunits; calcineurin A (a catalytic subunit) and calcineurin B (a regulatory calcium-binding subunit) of approximately 60 kDa and 20 kDa respectively (Li *et al.,* 2010; Rusnak and Mertz, 2000). The A subunit is composed of four different regions; the N-terminal region, the catalytic domain, the regulatory domain and the C-terminal region. The regulatory domain is further subdivided into the calcineurin B binding region, the calmodulin binding region and an auto-inhibitory peptide region (Figure 1.6) (Li *et al*., 2010).

There are three calcineurin A isoforms (alpha, beta and gamma), encoded by three different genes. The polypeptides produced by these genes have different N- and C-terminal tails but highly conserved amino acid sequences in the catalytic and regulatory domains (Molkentin, 2004). The isoforms alpha and beta are distributed throughout the body, while γ is more restricted to the brain and testis (Gooch *et al.,* 2004).

Calcineurin B is highly conserved, encoded by two genes which generate two different isoforms; Cn B1 which is expressed ubiquitously, and Cn B2 that is more localised in testis (Shou *et al*., 2015).

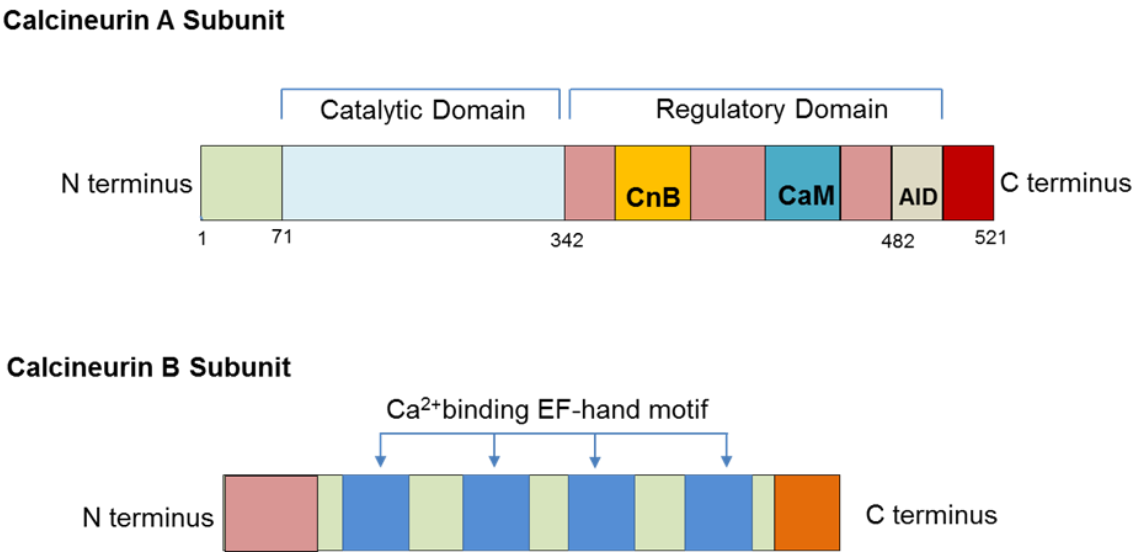


Figure 1.6 General structure of calcineurin (adapted from Shou *et al.,* 2015). The calcineurin heterodimer is formed by two subunits: calcineurin A (catalytic subunit) and calcineurin B (regulatory calcium-binding subunit). The A subunit is composed of four different regions; the N-terminal region, the catalytic domain, the regulatory domain and the C-terminal region. The regulatory domain is further subdivided into the calcineurin B binding region (CnB), the calmodulin binding region (CnM) and an auto-inhibitory peptide region (AID). Calcineurin B has a main region with four Ca^{2+}- binding EF-hand motifs.

Increments in the level of intracellular calcium promote the formation of calcium-calmodulin complexes that bind to the regulatory subunit of the heterodimer, activating calcineurin phosphatase activity (Schulz and Yutzey, 2004). The best known substrate of activated calcineurin is the family of NFAT (Nuclear Factor of Activated T-cells) transcription factors (Li *et al.,* 2010).

1.2.2 The NFAT protein family

The NFAT family of transcription factors consists of five members; NFAT1 (NFATc2/ NFATp), NFAT2 (NFATc1/NFATc), NFAT3 (NFATc4), NFAT4 (NFATc3/ NFATx) and NFAT5 (tonicity enhancer binding protein) (Mancini and Toker, 2009).

The NFAT proteins were first discovered in activated T lymphocytes, but later studies have shown that this family of proteins is ubiquitously expressed and plays an important

role in many pathophysiological processes such as cardiac development, tumour progression, cardiac hypertrophy, diabetes, vascular and neural development, etc (Crabtree and Olson, 2002).

Structurally, NFATs contain a region of high homology among all its members (NFAT homology region, NHR), a region which shows homology to the Rel protein (Rel homology region, RHR), and a C-terminal domain (Figure 1.7) (Shaul *et al.,* 2007).

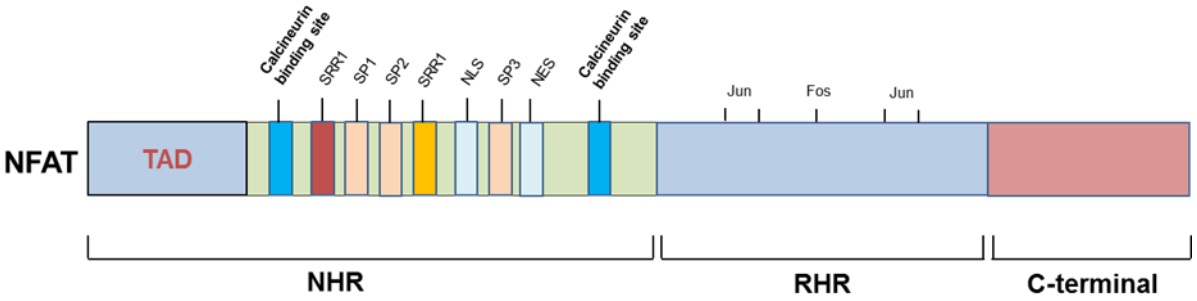


Figure 1.7 General structure of NFAT (adapted from Shou *et al*., 2015) NFAT proteins show a Rel-homology region (RHR), an NFAT homology region (NHR) and a C-terminal domain. The NHR contains an N-terminal transactivation domain (TAD), calcineurin binding sites, nuclear localisation sequence (NLS) and nuclear export signal (NES). It also contains several serine-rich motifs including SRR1, SRR2, SP1, SP2 and SP3. The RHR includes the DNA binding domain and sites for interaction with transcriptional binding partners such as Fos and Jun.

The NHR region is subdivided into an amino terminal transactivation domain (TAD) essential for the activity of the protein, calcineurin-binding sites and a nuclear localisation signal (NES) (Mancini and Toker, 2009; Li *et al.,* 2010). In the NHR region, we also find several intermediate serine-rich motifs called SRR1, SRR2, SP1, SP2, and SP3 (Hogan *et al.,* 2003). The RHR region contains the DNA binding domain and also recognition points for other transcription factors such as Fos and Jun that bind NFATs in certain circumstances (Shaul *et al.,* 2007) (Figure 1.7). NFATs1-4 are synthesized as constitutively phosphorylated proteins that are expressed in the cytoplasm of the cell. In response to increments in the intracellular calcium concentration, calcineurin becomes activated. Upon activation, calcineurin dephosphorylates NFAT1-4 transcription factors

promoting their translocation from the cytoplasm to the nucleus. Once in the nucleus NFAT1-4 bind to specific DNA motifs located in the promoter regulatory regions of target genes, switching on the transcription of these genes (Zaichuk *et al*., 2004; Ryeom *et al*., 2008; Armesilla *et al*., 1999). Nuclear NFAT1-4 are deactivated by rephosphorylation by specific kinases. Rephosphorylation of the protein induces a conformational change that results in exposure of a nuclear export sequence, triggering the movement of NFAT back to the cytoplasm (Beals *et al.,* 1997; Llang *et al.,* 2003).

1.2.3 Members of NFAT

1.2.3a NFAT1

Several studies are shown that NFAT1 is involved in the regulation of the immune response. *NFAT1*$^{-/-}$ mice have splenomegaly and hyperproliferation of both B and T cells (Hodge *et al.,* 1996). Horsley *et al.*, 2001 demonstrated that NFAT1 plays a role in the regulation and growth of skeletal muscle (Horsley *et al.,* 2001).

1.2.3b NFAT2

NFAT2 has been shown to play an important role in cardiac development, specifically in valve formation. In a study by De la Pompa *et* al., 1998 the authors showed that *NFAT2*$^{-/-}$ mice are embryonic lethal at day E14.5 due to lack of development of the cardiac valves and septa (De la Pompa *et* al., 1998). Another study using *NFAT2*$^{-/-}$ mice also revealed a pathological phenotype of congestive heart failure between days E13.5 to E17.5 due to loss of aortic and pulmonary valve formation (Ranger *et al.,* 1998a).

1.2.3c NFAT3

Deletion of NFAT3 in *NFAT3*$^{-/-}$ mice had no effect on embryonic development. However, when combined with *NFAT4*$^{-/-}$, the double knock out (DKO) *NFAT3*$^{-/-}$*; NFAT4*$^{-/-}$ shows embryonic development defects starting around day E10.5. These isoforms of NFAT are also required for recruitment of smooth muscle cells to the vessels to enhance stability (Graef *et al.,* 2001).

1.2.3d NFAT4

Using NFAT knockout mice, NFAT4 has been shown to play an important role in the stabilisation of the vasculature and functionality of the developing heart. It has also been shown that NFAT4 is implicated in the regulation of skeletal muscle growth at E17.5 (Graef *et al.,* 2001). Double knockout of NFAT1 and 4 showed that these proteins have a role in the negative regulation of the immune response. NFAT1/4 DKO mice show an increase in mast cells and eosinophils in the spleen and lymph nodes (Ranger *et al.,* 1998a).

1.2.3e NFAT5

NFAT5 is included in the NFAT family of transcription factors because it has similar structural features. However, it differs from other NFATs in its mechanisms of activation. In contrast to members NFAT1-4 that are synthesised as phosphorylated proteins located in the cytoplasm of the cells and activated by calcineurin-dependent dephosphorylation and subsequent nuclear translocation, NFAT5 activation is independent of calcineurin, and a high proportion of NFAT5 is constitutively localised in the nucleus, although cellular activation is required for total NFAT nuclear localisation (Aramburu *et al.,* 2006).

Functionally, NFAT5 activates a cellular gene expression programme in response to osmotic stress (Aramburu *et al.,* 2006; Lopez-Rodriguez *et al,* 2001; Drews-Elger *et al.,* 2009). Additionally, Buxade *et al.,* 2012 have recently reported that NFAT5-deficient mice show a remarkable susceptibility to *Leishmania major* infections (Buxade *et al.,* 2012) revealing NFAT5 as a crucial inducer of the expression of genes essential for antimicrobial immunity.

Although the role of NFAT1-4 members in endothelial cell biology and, in particular, endothelial cell angiogenesis has been extensively studied (Armesilla *et al.,* 1999;

Hernandez *et al.,* 2001; Urso *et al*. 2011) whether NFAT5 regulates the expression of endothelial genes during angiogenesis has not been explored so far.

1.2.4 Inhibitors of the calcineurin/NFAT pathway

1.2.4a Cyclosporin A and FK506

Cyclosporin A (CsA) and tacrolimus (FK506) are two effective immunosuppressive drugs widely used in organ transplantation and dermatology. Application of these compounds in basic research has contributed to study the molecular mechanisms that regulate calcineurin-dependent signalling pathways (Sieber and Baumgrass, 2009). These two immunosuppressive drugs bind to small intracellular proteins; cyclophilin A and FK506 binding protein (FKBP12) respectively (Li *et al*, 2011; Martínez-Martínez and Redondo, 2004). These inhibitory complexes interact with active sites of calcineurin preventing its interaction with NFAT (Ruff and Leach, 1995; Loh *et al*., 1996). Clinical use of CsA and FK506 is associated with severe side effects in the patient, including kidney damage, renal failure, hypertension and gastrointestinal problems, therefore restricting their use in treating chronic diseases (Martínez-Martínez and Redondo, 2004).

1.2.4b Calcineurin Homologous Protein (CHP)

Lin *et al.,* 1999 proved that calcineurin is also the target of a novel Ca^{2+}-binding protein called calcineurin homologous protein (CHP) which is ubiquitously expressed. CHP shares a high percentage of sequence homology with CnB and with CaM (65% and 59% respectively). The authors demonstrated that overexpression of CHP in Jurkat and HeLa cells blocks nuclear translocation of NFAT in response to PMA and ionophore stimulation. Furthermore, overexpression of CHP inhibits calcineurin phosphatase activity by preventing the assembly of calcineurin with CnB and CaM. This data strongly demonstrated that CHP attenuates the activity of the calcineurin/NFAT signalling pathway (Lin *et al.,* 1999).

1.2.4c A-Kinase-Anchoring Protein (AKAP79)

AKAP79 is an approximately 79 kDa protein kinase A anchoring protein, that interacts with calcineurin and inhibits its activity (Martínez-Martínez and Redondo, 2004). It is mainly expressed in T-cells and neurons (Kashishian *et al*, 1998). Kashishian *et al*, 1998 reported that overexpression of AKAP79 inhibits NFAT activation induced by PMA and ionophore stimulation (Kashishian *et al*, 1998). Additionally, co-expression of calcineurin and AKAP79 showed translocation of calcineurin from the cytoplasm to the membrane confirming the interaction between the two proteins (Dell'Acqua *et al*, 2002).

1.2.4d Cabin 1/Cain

Cabin1 (calcineurin binding)/ cain (calcineurin inhibitor) is an ubiquitously expressed 230 kDa phosphoprotein that interacts with calcineurin and inhibits its activity (Lai *et al*, 1998; Sun *et al*, 1998). Binding of Cabin 1 to calcineurin is dependent on both calcium signalling and PKC activation. As with AKAP79 and CHP, overexpression of Cabin 1 in T-cells downregulates calcineurin phosphatase activity and consequently attenuates NFAT activation (Sun *et al*, 1998).

1.2.4e Regulator of calcineurin 1 (RCAN1)

The human *RCAN1* gene, also referred to Down syndrome candidate region 1 (DSCR1), myocyte-enriched calcineurin interacting protein 1 (MCIP1), Adapt 78, and calcipressin, is located on chromosome 21, near the portion of the Down Syndrome Critical Region (Fuentes *et al*., 1997; Davies *et al*, 2007). This gene is comprised of 7 exons. Exons 1-4 are alternatively spliced, resulting in two main isoforms RCAN1-1 and RCAN1-4. Exon 1 gives rise to the isoform RCAN1.1 (Genescà *et al.,* 2003). Exon 2 does not have a methionine start site important for translation, and exon 3 encodes for only three amino acids (Yang *et al.,* 2000). Exon 4 gives rise to isoform RCAN1.4 which contains 15 binding sites for NFATs in the regulatory region that controls expression of isoform 1.4 (Holmes *et al.,* 2010; Genescà *et al.,* 2003).

Several studies have shown VEGF-stimulated induction of RCAN1.4 in a variety of endothelial cells. Only RCAN1.4 is upregulated in response to VEGF, while RCAN1.1 is unaffected (Iizuka *et al*., 2004; Yao and Duh, 2004). VEGF-mediated upregulation of RCAN 1.4 is blocked by inhibitors of calcineurin A such as CsA, FK506, or an anti-VEGFR-2 monoclonal antibody (Chan *et al*., 2005). Maximal induction of RCAN1.4 expression in human dermal microvascular endothelial cells has been shown to require activation of the calcineurin/NFAT pathway and the PKC-delta pathway (Holmes *et al.,* 2010).

Overexpression of RCAN1 protein inhibits calcineurin in the nanomolar range, acting in a similar way to the pharmacological agents FK506 and cyclosporin A (Chan *et al.,* 2005).

Over-expression of RCAN1.4 in an animal human tumour xenograft model showed reduced vascular density and melanoma growth, highlighting that induction of RCAN1.4 results in a negative feed-back mechanism that subsequently inhibits the calcineurin/NFAT pathway (Minami *et al.,* 2004).

Analysis of endothelial cell function in cells undergoing siRNA-mediated silencing of *RCAN1* showed that lack of RCAN1 resulted in disrupted VEGF-mediated tubular morphogenesis and cell migration (Holmes *et al.,* 2010). Endothelial cells isolated from RCAN1$^{-/-}$ mice show increased apoptosis (Ryeom *et al*., 2008) suggesting that RCAN1.4 functions to negatively regulate calcineurin-dependent apoptosis in endothelial cells and this role of RCAN1 is essential for successful angiogenesis.

1.2.4f Plasma membrane calcium ATPase (PMCA)

Plasma membrane calcium ATPases (PMCAs) have been recently characterised as novel regulators of signal transduction pathways (including the calcineurin/NFAT signalling pathway) via interaction with its partner proteins. Several studies have demonstrated a role for PMCA as an inhibitor of the calcineurin/NFAT pathway in breast

cancer cells (Holton *et al*., 2007), cardiomyocytes (Wu *et al*., 2009) and VEGF-stimulated endothelial cells (Baggott *et al*., 2014). Ectopic expression of PMCA4 in HEK293 cells was shown to downregulate NFAT activation triggered by stimulation of the cells with PMA and calcium ionophore (Buch *et al*., 2005). A similar result was observed in cells overexpressing recombinant PMCA2 (Holton *et al.,* 2007). We have also reported that PMCA4 is an endogenous inhibitor of calcineurin-dependent angiogenesis (Baggott *et al*., 2014) and for that reason we have selected PMCA4 for the experiments conducted in this thesis. Because of the relevance of PMCA proteins in the work carried out in this thesis, I will describe in the following sections the main features of the PMCA family of proteins formed by four members (PMCA1-4).

1.3 Plasma membrane calcium ATPase pump (PMCA)

PMCAs belong to the large super family of P-type ATPase calcium transporter membrane proteins which also includes, Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA), the sodium-potassium ATPase (Na^{2+}/K^{+}-ATPase), hydrogen-potassium ATPase (H^{+}/K^{+}-ATPase) and others (Verma *et al.,* 1988). PMCAs transport calcium from the cytoplasm to the extracellular environment using the energy released by hydrolysis of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) (Palmgren and Nissen, 2011). PMCAs extrude calcium with low capacity but high calcium affinity (Carafoli *et al*., 2000).

1.3.1 Calcium transport by PMCAs

PMCAs can be found in two conformational states known as E1 and E2 (Figure 1.8) (Carafoli E, 1991). In the E1 state, the conformation of PMCAs expose a high affinity calcium binding site to the cellular cytosol allowing incorporation of calcium into the pump. Binding of calcium leads to phosphorylation of the protein in an aspartic residue (conserved in all PMCA isoforms) in a process that requires hydrolysis of ATP. This phosphorylated intermediate is known as E1-P (Figure 1.8). The conformation of PMCA

at E1-P is not stable, and thus, in a mechanism promoted by Mg^{2+}, the pump undergoes a conformational change that results in a more stable intermediate called E2-P. The conformational change that mediates the E1-P to E2-P transition places the calcium binding site on the external side of the plasma membrane, effecting calcium translocation and its release into the extracellular medium (Figure 1.8). E2-P is then converted into a dephosphorylated intermediate E2 that will finally revert to the original state E1, in a transition accelerated by Mg^{2+}, completing the cycle (Figure 1.8) (Adamo *et al*., 1990).

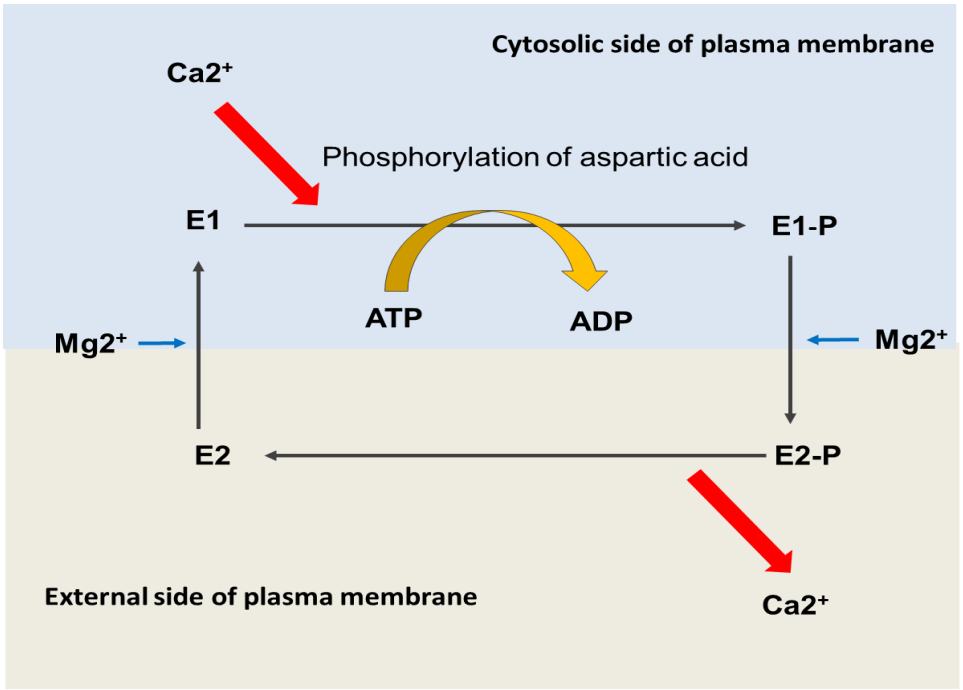


Figure 1.8 Model depicting the molecular events implicated in PMCA-mediated calcium transport through the plasma membrane (adapted from Adamo *et al*., 1990)**.** E1, PMCA at conformational state 1. E1-P, phosphorylated intermediate of PMCA in the conformational state E1. E2-P, phosphorylated intermediate of PMCA in the conformational state E2. E2, PMCA at conformational state E2.

1.3.2 Structure of PMCAs

The PMCA protein is formed by a single polypeptide chain. The putative structure of PMCAs consists of ten transmembrane (TM) segments linked by five extracellular short loops with the majority of the protein being cytosolic (Figure 1.9) (Kindmark *et al*., 1994). The cytosolic part of PMCAs is further subdivided into four main domains and two small loops (Figure 1.9).

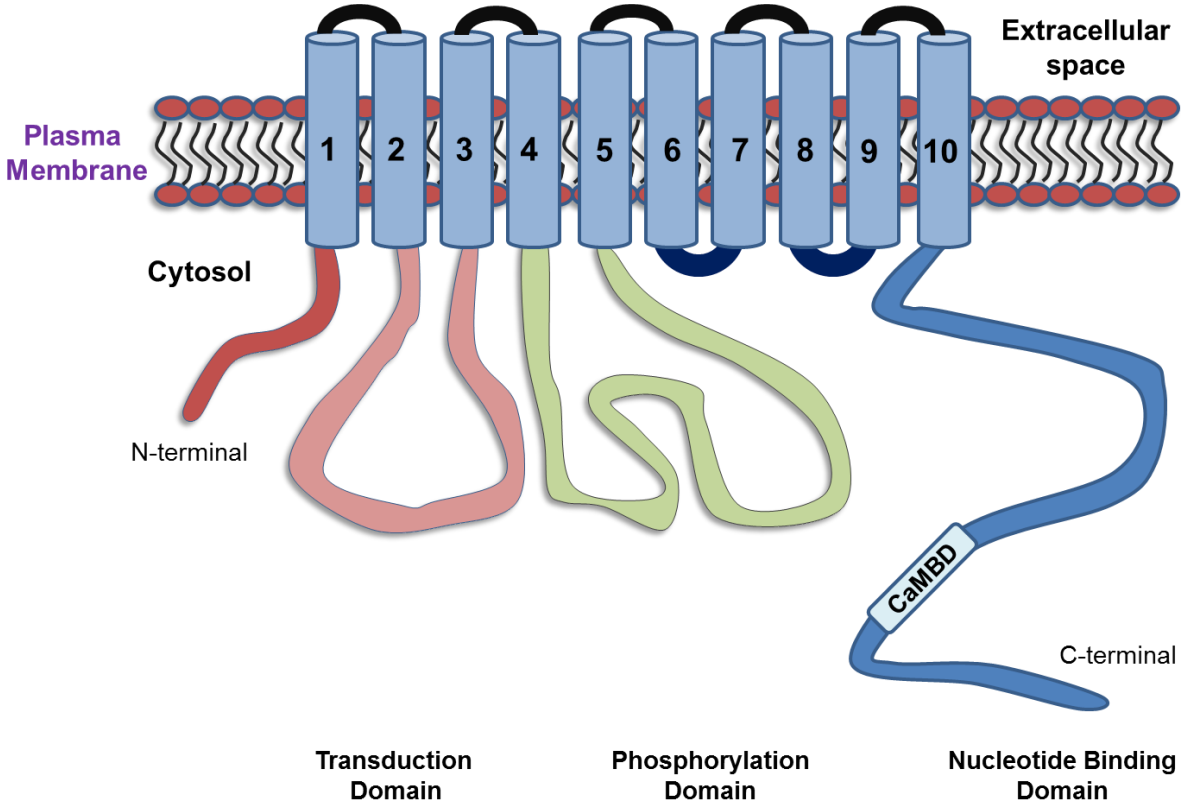


Figure 1.9 The membrane topology of PMCAs (adapted from Di Leva *et al* 2008; Strehler and Zacharias, 2001) PMCAs consist of ten transmembrane domains with five extracellular and six intracellular domains (N-terminal, intracellular domain between TM2 and TM3 (Transduction domain), intracellular domain between TM4 and TM5, two small intracellular loops and C-terminal region that contains a binding site for calmodulin (CaMBD).

The first cytoplasmic domain is the N-terminal region which contains 80-90 amino acids. The second domain corresponds to an intracellular loop located between TM2 and TM3 which is also called the transduction domain. The third one is a large intracellular loop

located between TM4 and TM5 which corresponds to the catalytic domain of the protein (Figure 1.9). This domain contains the aspartic residue phosphorylated in the E1-P intermediate state of the calcium translocation cycle and the binding site for ATP. The catalytic domain is followed by two small intracellular loops connecting TM6 with TM7 and TM8 with TM9. The C-terminal domain harbours a binding site for calmodulin (CaMBD) (regulatory site) which is involved in the regulation of PMCA activity (Figure1.9) (Di Leva *et al.,* 2008).

1.3.3 PMCA isoforms and tissue distribution

As previously mentioned, there are four isoforms of PMCA (PMCA1-4) encoded by four distinct genes (Di Leva *et al.,* 2008). RNA alternative splicing in two possible splicing sites (site A located in the first intracellular loop, and site C located in the COOH-terminal tail) generates more than 20 PMCA splice variants (Strehler and Zacharias, 2001). Differences in the amino acid sequences between these four isoforms are found mostly in the N-terminal and C-terminal domains of the protein (Strehler and Zacharias, 2001).

1.3.3a PMCA1

PMCA1 is widely distributed in all tissues but most highly expressed in brain, lungs and intestine (Di Leva *et al.,* 2008). PMCA1 is believed to have a major ‘‘housekeeping’’ or developmental function as *PMCA1*$^{-/-}$ mice do not survive past embryonic development (Okunade *et al*., 2004). PMCA1 has recently been reported to have a critical role in the regulation of blood pressure as heterozygous *PMCA1*$^{+/-}$ mice present elevated blood pressure (Fujiwara *et al.,* 2014). Consistently with this data, mice with tissue-specific deletion of PMCA1 in vascular smooth muscle cells exhibit elevated systolic blood pressure. Furthermore, femoral arteries from these mice present enhanced contractility to phenylephrine (Kobayashi *et al*., 2012). Importantly, single nucleotide polymorphisms in the *PMCA1* gene have a strong association with hypertension in different human

ethnic groups (Tabara *et al*., 2010; Ganesh *et al.,* 2013, Wang *et al*., 2012; Xi *et al*., 2012). All these data strongly suggests a pivotal role for PMCA1 in blood pressure regulation, although the molecular mechanisms underlying this function of PMCA1 are still largely unknown.

1.3.3b PMCA2

PMCA2 has a more restricted tissue distribution and is localised to the brain, liver, uterus, kidney, cochlear hair cells and lactating mammary glands. PMCA2 plays an essential role in neuronal calcium homeostasis (Kurnellas *et al.*, 2006). It also has a critical role in the physiology of both the vestibular and auditory systems as *PMCA2*$^{-/-}$ mice are profoundly deaf and show difficulties in maintaining balance (Kozel *et al.,* 1998). During lactation there is a significant up-regulation in the expression of PMCA2 in mammary glands (Reinhardt *et al.,* 2004). This up-regulation is connected with an increase of calcium levels in milk, suggesting that PMCA2 is involved in calcium transport from blood to milk during lactation. Supporting this idea, milk from PMCA2-null mice contains 60% less calcium than milk produced by heterozygous or wild-type mice (Reinhardt *et al.,* 2004). PMCA2 not only plays an important role in mammary gland physiology but also in breast cancer progression. PMCA2 expression is clearly upregulated in breast cancer cell lines (Lee *et al.,* 2005). Several lines of evidence have reported that PMCA2 protects breast cancer cells from apoptosis and, in this way, it contributes to the progression of breast cancer (VanHouten *et al*., 2010; Peters *et al.,* 2016)

1.3.3c PMCA3

PMCA3 is predominantly present in choroid plexus and in skeletal muscle (Strehler and Zacharias, 2001). However, its physiological function is still unclear as PMCA3 knockout models have not been determined to date (Prasad *et al.,* 2004).

1.3.3d PMCA4

PMCA4 is ubiquitously expressed. Analysis of mice deficient in PMCA4 are starting to unravel the function of this isoform in specific tissues and cell types. For example, *PMCA4*$^{-/-}$ mice have been reported to be infertile due to defects in sperm motility (Schuh *et al.,* 2004). Several studies using PMCA4-deffficent mice have also shown that PMCA4 plays an important role in the pathophysiology of the heart. PMCA4 has been implicated in the regulation of the inotropic response of cardiomyocytes to β-adrenergic stimuli (Oceandy *et al.,* 2007; Mohamed *et al.,* 2009). Deregulation of PMCA4 function has been linked as well to cardiac pathology. Several groups have investigated the role of PMCA4 in the progression of cardiac hypertrophy using mice deficient in PMCA4, but the outcomes of their studies have revealed contradictory results. Whereas Wu *et al.,* 2009 reported that PMCA4 antagonizes cardiac hypertrophy induced by transverse aortic constriction (Wu *et al.,* 2009), Mohamed *et al.,* 2016 have recently shown that deletion of PMCA4 (systemically or specifically in fibroblasts) attenuates the hypertrophic response in the heart induced by this stimulus. In this work, the authors also demonstrate that specific ablation of PMCA4 in cardiomyocytes does not produce this effect. Animal models of cardiomyopathy-induced cardiac hypertrophy have also shown that loss of PMCA4 blocks the development of cardiac hypertrophy (Prasad *et al.*, 2014). Using gain-of-function mouse models Ocenday *et al*., reported that overexpression of PMCA4 in cardiomyocytes increases cardiac hypertrophy induced by chronic stimulation with isoproterenol (Ocenday *et al., 2007).* The reason for discrepancies in these data with the initial observation reported by Wu *et al* is not understood at present.

PMCA4 has also been implicated in the regulation of VEGF induced angiogenesis *in vivo* and *in vitro* (Baggott *et al.,* 2014). *PMCA4*$^{-/-}$ mice present enhanced post-ischemic hindlimb perfusion 5 and 14 days after surgery compared to wild type littermates

(Baggott *et al*., 2014). It is this data that prompted me to formulate the hypothesis tested in this thesis and that will be explained later in detail.

1.3.4 Activation of PMCA activity

PMCA has poor affinity for calcium in an unstimulated state and needs to be activated to increase its affinity for calcium (Cheung *et al.,* 1980). A number of mechanisms including modification by kinases, interaction with proteins, cleavage, and self-association increase the affinity of PMCA for calcium (Di Leva *et al.,* 2008). The traditional endogenous activator of the pump is calmodulin, which also acts as a regulator of other calcium-dependent enzymes (James *et al*., 1988; Vorherr *et al.,* 1990). PMCAs contain a calmodulin binding domain (CaMBD) located in the C-terminus of the protein (Figure 1.10). This domain interacts with the first intracellular loop (located between TM2 and TM3) and with the large catalytic domain of the pump, acting as an auto-inhibitory domain that maintains the protein in an inactive state. This conformation of the protein is known as the ‘’closed’’ conformation (Figure 1.10). Increments in the level of intracellular calcium prompt the association of calcium-calmodulin complexes to the CaMBD, displacing the interactions with other regions of the PMCA and activating the pump (Figure 1.10) (James *et al*., 1988; Vorherr *et al.,* 1990). This confirmation of the protein is called the ‘’open’’ conformation. PMCAs fluctuate between the ‘’open’’ and ‘’closed’’ (active-inactive) conformations depending on the concentration of intracellular calcium. Enyedi *et al.,* 1989, proved that the CaMBD acts as an auto-inhibitory mechanism by using a mutant of PMCA4 that lacks the last 120 residues (including the 28-aminoacids of the calmodulin binding inhibitory domain). They showed that this mutant was constitutively active, independently of calcium and calmodulin, and was 10 times more active than the wild-type protein (Enyedi *et al.,* 1989).

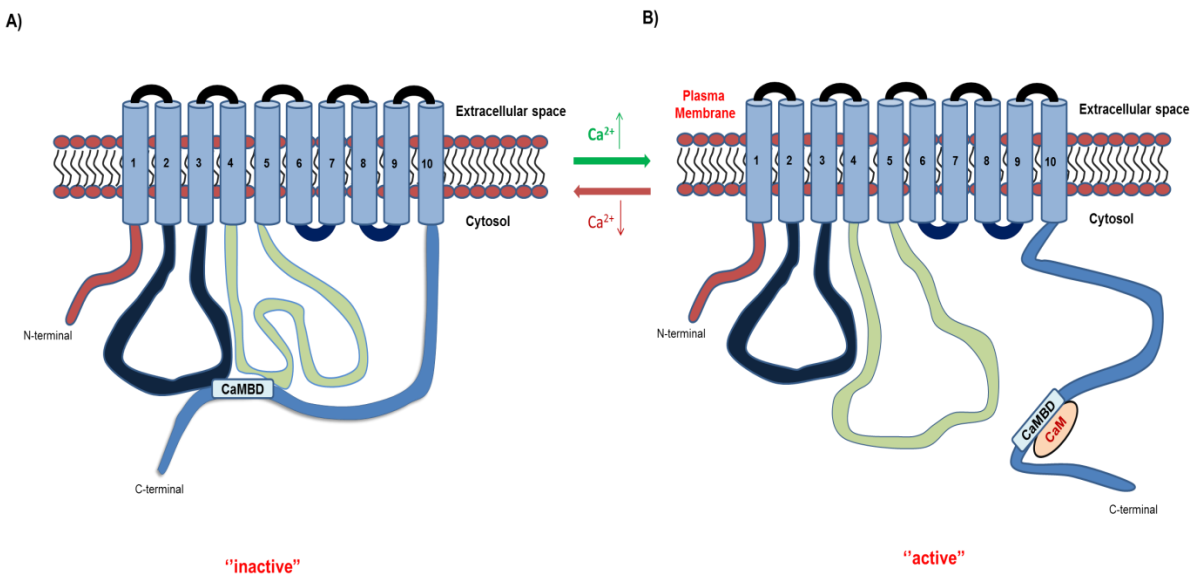


Figure 1.10 Activation of PMCAs (adapted from Di Leva *et al* 2008). A) In an inhibited state the calmodulin binding domain of the pump interacts with the intracellular domains. B) In an activated state, calmodulin binds to the calmodulin binding site releasing the interaction of the auto-inhibitory domain, which consequently activates the pump.

The activity of PMCAs is also regulated by acid phospholipids. Specifically, phosphatidylinositol 4,5 bisphosphate has been shown to activate PMCAs in human erythrocytes by interacting with two binding domains; one is located to the intracellular loop between TM2 and TM3, and the second in the C-terminal at the CaMBD site (Niggli *et al.,* 1981; Brodin *et al.,* 1992). The mechanism by which acidic phospholipids activate PMCAs has not been thoroughly studied yet.

Under certain conditions, PMCAs are phosphorylated by a variety of kinases including protein kinase A (PKA), protein kinase C (PKC), and cAMP-dependent protein kinase and GMP-dependent protein kinase (Monteith, *et al.,* 1999). PKA directly phosphorylates PMCAs at a serine residue which is located downstream of the CaMBD. PKC also activates the pump by phosphorylating a threonine residue which is located in the CaMBD and thus modulates the auto-inhibitory conformation of the pump (James *et al.*, 1989).

1.3.5 PMCA interacting partner proteins

In addition to their role as calcium transporters, PMCAs have recently emerged as novel regulators of signal transduction pathways via interaction with a variety of cytoplasmic cellular proteins (Holton *et al.,* 2010). These interactions involve different PMCA intracellular domains and regulate the activity of both PMCA and the partner proteins. Interactions between PMCAs and cytoplasmic partner proteins that I describe below are illustrated in Figure 1.11.

A small acidic protein, 14-3-3e, interacts with the N-terminal region of PMCA. This interaction is isoform specific. PMCA1, PMCA3, and PMCA4 have been reported to interact with 14-3-3e but not PMCA2 (Rimessi *et al*., 2005; Linde *et al*., 2008).

Several partner proteins have been found to interact with the catalytic intracellular domain of the pump. In this sense, Buch *et al.,* 2005 showed that ectopically expressed PMCA4b interacts with calcineurin in HEK293 cells via interaction to this domain of PMCA4 (Buch *et al*., 2005). The authors demonstrated that this interaction resulted in a significant reduction in the activity of the calcineurin/NFAT pathway. This intracellular region of PMCA has also been found to interact with endogenous calcineurin in other cellular types such as breast cancer cells (Holton *et al.,* 2007). Interestingly, the interaction of PMCA with calcineurin in breast cancer cells is isoform specific. Calcineurin strongly binds to the PMCA2 isoform but interacts very weakly with PMCA4 and no interaction has been observed with PMCA1 (Holton *et al.,* 2007). Disruption of the interaction between endogenous PMCA2 and calcineurin in breast cancer cells by over-expression of the PMCA2-interaction domain triggers calcineurin-mediated apoptosis and enhances paclitaxel-mediated cytotoxicity (Baggott *et al*., 2012). PMCA4/calcineurin interaction has also been found in other cell types such as cardiomyocytes (Wu *et al.,* 2009), PC12 cells (Kosiorek *et al.,* 2011), and endothelial cells (Holton *et al.,* 2010).

The catalytic loop of PMCAs has also been reported to interact with endothelial nitric oxide synthase (eNOS) in endothelial cells (Holton *et al.,* 2010). Ectopic expression of PMCA in endothelial cells increases the phosphorylation of the residue Thr-495 of eNOS leading to a significant decrease in the production of nitric oxide (NO) in response to acetylcholine or ionophore stimulation of the cells (Holton *et al.,* 2010). These studies highlighted that eNOS activation is negatively regulated by endothelial PMCAs.

Another cytoplasmic PMCA4 partner protein that has been identified to interact with the large intracellular catalytic loop of PMCA4 is RASSF1 (Armesilla *et al.,* 2004). RASSF1 is a tumour suppressor protein that is implicated in Ras-mediated apoptosis (Vos *et al.,* 2000). There are two major isoforms of RASSF1, RASSF1A and RASSF1C, and both have been shown to interact with PMCA4b in HEK293 cells through association with the region encompassing amino acids 652-748 of PMCA4, located in the catalytic intracellular loop (Armesilla *et al*., 2004).

α-1 syntrophin has also been identified as an interaction partner of PMCA4b and PMCA1b in HEK293, cells via binding to the big intracellular loop of the pump (Williams *et al*., 2006). Interestingly, syntrophin interacts with PMCA4 and nNOS simultaneously to form a macromolecular complex (Williams *et al.,* 2006).

Regarding interactions with the C-terminal tail of PMCAs, it has been demonstrated that this region of the pump holds a post-synaptic density, Drosophila disc large tumour suppressor and Zona occludens-1 (PDZ) binding domain that interacts with cytosolic proteins containing PDZ domains (Di leva *et al*., 2008). The interaction of PDZ-containing proteins with PMCAs is isoform-specific in many cases, for example MAGUK (membrane-associated gunylate kinase) proteins contain three PDZ domains that interact with PMCA isoforms 2b and 4b (DeMarco S and Strehler E, 2001). Likewise, the PDZ domain-containing protein NHERF2 (Na^+/H^+ Exchange Regulatory Factor 2)

interacts with the C-terminal tail of PMCA2b but not PMCA4b (DeMarco *et al.,* 2002). Other cytoplasmic proteins reported to interact with the C-terminal tail of PMCA4b are CASK (calcium/calmodulin-dependent serine protein kinase) (Suhuh *et al*., 2003), PISP (PMCA-interacting single-PDZ domain) (Goellner *et al.,* 2003), Ania-3 (Sgambato-Faure *et al*.., 2006), CLP-36 (Bozulic *et al.,* 2007), and nNOS (neural Nitric Oxide Synthase) (Schuh *et al.,* 2001).

Although the functional significance of the interaction between PMCAs and partner proteins has not been completely elucidated in all cases, a large group of data supports that PMCAs regulate the activity of the partner proteins by establishing these interactions, and, in this way, PMCAs exert a pivotal role as modulators of the signal transduction pathways where the partner proteins are involved. In agreement with this concept, the interaction of PMCAs with eNOS or nNOS results in inhibition of nitric oxide production and thus, downregulation of NO-dependent signalling (Holton *et al*., 2012; Schuh *et al* 2001; Oceandy *et al*., 2007; Mohamed *et al*., 2009). In the same context, the interaction PMCA/calcineurin inhibits calcineurin activity leading to a downregulation of the calcineurin-NFAT signalling pathway (Buch *et al.,* 2005; Holton *et al*., 2007; Wu *et al.,* 2009; Holton *et al*., 2010a; Holton *et al*., 2010b; Kisorek *et al*., 2011; Baggott *et al.*, 2012; Baggott *et al.,* 2014). Given the broad spectrum of implications for calcineurin signalling in the pathophysiology of the cell (Crabtree and Olson, 2002), the functional consequences of PMCA-mediated regulation of calcineurin are far reaching. Our group has recently reported that PMCA4 inhibits VEGF-driven angiogenesis via interaction with calcineurin in endothelial cells (Baggott *et al.,* 2014). Thus, modulation of PMCA4 activity and, in turn, modulation of calcineurin signalling in endothelial cells, might be used with therapeutic purposes to treat patients suffering with diseases associated with abnormal angiogenesis. One of the lines of research under investigation in our laboratory focuses on the identification of selective inhibitors of PMCA4 function,

and the work presented in this thesis has examined the consequences of pharmacological-mediated inhibition of PMCA4 on VEGF-driven angiogenesis.

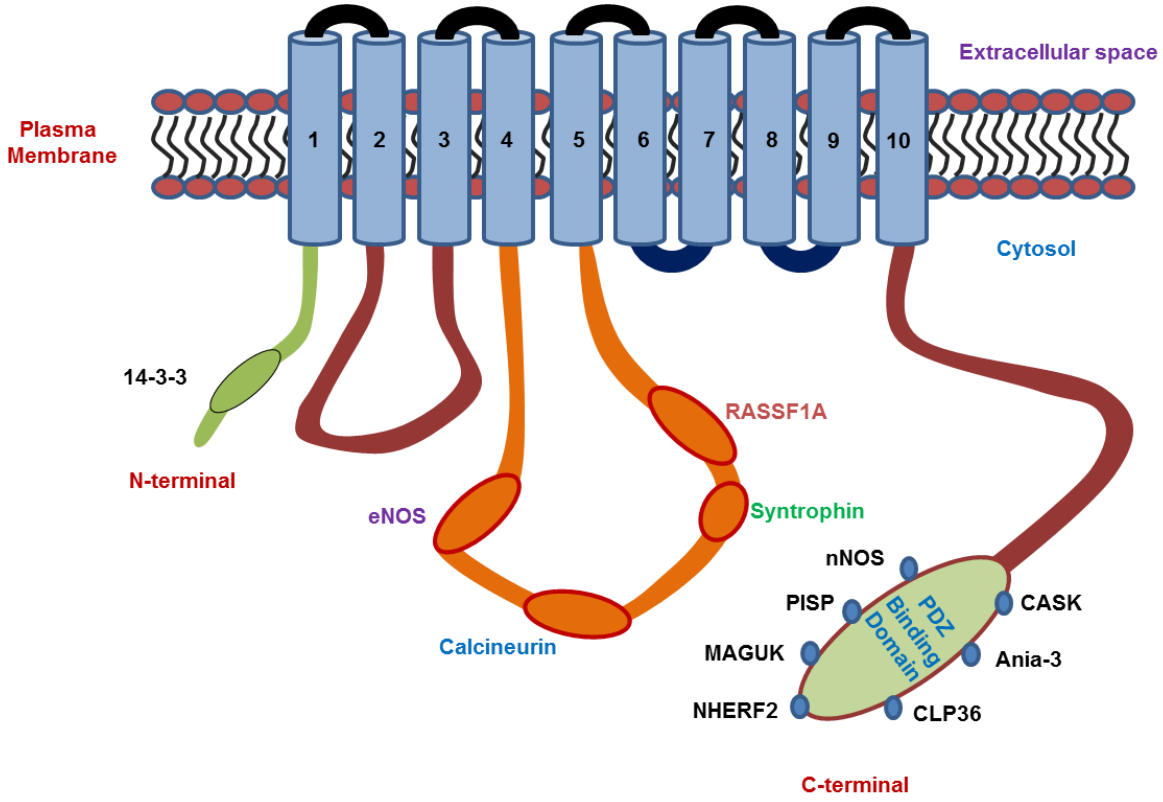


Figure 1.11 PMCA interacting partner proteins (adapted from Di Leva *et al.,* 2008). eNOS, endothelial nitric oxide synthase; RASSF1, Ras-associated factor 1; nNOS, neuronal nitric oxide synthase; CASK, calcium/calmodulin-dependent serine protein kinase; MAGUK, membrane-associated gunylate kinase; PISP, PMCA-interacting single-PDZ domain; NHERF2, Na^{+}/H^{+} Exchange Regulatory Factor 2; PDZ, post-synaptic density, Drosophila disc large tumour suppressor and Zona occludens-1.

1.3.6 PMCA inhibitors

Initially, lanthanium (La^{3+}) and vanadate were used to inhibit PMCA4. They are general inhibitors of all P-type ATPases as they both inhibit ATPase activity by competing for the ATP binding site in the catalytic domain of all P-type ATPases (Quist *et al.*, 1975; Cantley *et al.,* 1977; Barrabin *et al.,* 1980). Therefore, lanthanium or vanadate could not

be used as a specific inhibitor for PMCA4 in this study as they would act on any P-type ATPase.

As PMCA4 ATPase activity is calmodulin dependent, it can therefore be inhibited by calmodulin antagonists, such as calmidazolium, which inhibits PMCA4 by direct binding to calmodulin, or by interacting with its binding sites (Gietzen *et al.,* 1982). Calmidazolium could not be used as a PMCA4 specific inhibitor as it would also inhibit the other members of the PMCA family and all calmodulin-dependent proteins such as calmodulin-dependent protein kinase (CaMK), calcineurin, calmodulin-dependent phosphodiesterase (PDE) and others (Sunagawa *et al.,* 1998; Mukai *et al.,* 1991).

Polypeptides called caloxins have been used to inhibit PMCA by binding to the extracellular domains of the pump. The extracellular sequences of all PMCA isoforms are extremely well conserved and it is therefore difficult to achieve inhibition of a specific isoform. Caloxin 1c2 has shown high specificity to PMCA4, but it also inhibits other isoforms with lower affinity (Strehler *et al.,* 2007; Szewczyk *et al.,* 2007; Pande *et al.,* 2011).

In many studies eosin and its analogue carboxyeosin have been reported as potent inhibitors for all PMCA isoforms; they act by interfering with the binding of ATP to a conserved site found in all ATPases (Gatto *et al*., 1993; Shmigol *et al*., 1998).

In summary, although all these compounds inhibit PMCA4, they did not show more potency in inhibiting PMCA4 activity compared with other ATPases. However, Mohamed *et al.,* 2013, recently identified a small molecule which strongly inhibits PMCA4 and no other PMCA isoforms or other ATPases (Mohamed *et al.,* 2013). To identify this molecule the authors screened a library of 1280 medically optimised drug-like molecules against PMCA4 activity and found that aurintricarboxylic acid (ATA) has a high inhibitory effect for PMCA4 at a very low concentration (Mohamed *et al.,* 2013). ATA is a polyaromatic carboxylic acid derivative with a molecular weight of 422.345

g/mol (Figure 1.12). ATA easily polymerizes in aqueous solution forming a stable free radical (Kuban-Jankowska *et al.,* 2016). Although ATA has been reported to be soluble in aqueous solutions (Green, 1990), the solubility of ATA in organic solvents is around ten times higher than in water (Green, 1990). Cells in culture were treated with a minimal volume of ATA solution to avoid dilution of growth factors in the medium that could result in damage of cells. To achieve this, a very high concentrated stock of ATA was prepared. To maximize solubility of ATA in this concentrated stock DMSO was used as a solvent instead of water. As DMSO was used as a vehicle in the preparation of our stock solution, control cells in this work were treated with DMSO to be sure that any observed effects were due to the action of ATA and not to interference caused by the vehicle.

As ATA has been shown to inhibit the calcium extrusion activity of PMCA4 strongly and selectively at concentrations that do not exert any effect on the activity of PMCA1 or other major calcium pumps (such as SERCA and Na^+/K^+ ATPase) (Mohamed *et al.,* 2013) and PMCA4 has been reported to regulate the calcineurin/NFAT pathway, we decided to investigate in this work whether ATA-mediated inhibition of PMCA4 enhances the activity of the calcineurin/NFAT pathway and thus, calcineurin-dependent angiogenesis. Recently ATA was shown to inhibit the activity of PMCA4 *in vivo* using a mouse model of cardiac hypertrophy (Mohamed *et al.*, 2016). In this work, the authors demonstrated that injection into mice of ATA at 5 mg/kg/day for 2 weeks, reversed cardiac hypertrophy without causing any harmful side-effects on the animals (Mohamed *et al.*, 2016). Likewise, Zhang *et al.,* 2013, demonstrated the potential of using ATA at 20 mg/kg/day to reduce experimental autoimmune encephalomyelitis in mouse animal models without observing any toxic effects (Zhang *et al.,* 2013).

These studies indicate that the effects of ATA in pathological conditions can be tested *in vivo* using pre-clinical animal models. However, if this compound is to be translated into clinical applications in humans, ADMET studies need to be carried out to assess the feasibility of its use or whether more specific, non-toxic versions of ATA are required.

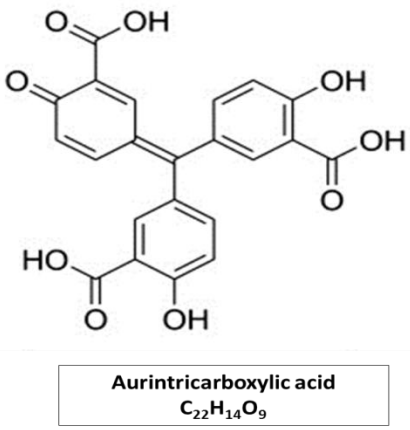


Figure 1.12 Structure of aurintricarboxylic acid (adapted from Kuban Jankowska *et al.,* 2016). Structure of aurintricarboxylic acid (ATA) showing chemical groups and atomic formula. ATA has been described as a strong inhibitor of PMCA4 calcium extrusion activity.

CHAPTER 2

Aims and Hypothesis

2. Aims and hypothesis

VEGF-mediated activation of the calcineurin/NFAT signalling pathway plays a pivotal role in the regulation of pathological and physiological angiogenesis (Armesilla *et al.,* 1999; Hernandez *et al.,* 2001). Our group has previously demonstrated that PMCA4 downregulates the activation of the calcineurin/NFAT pathways in endothelial cells stimulated with VEGF and, in doing so, inhibits VEGF-induced pro-angiogenic signalling (Baggott *et al.,* 2014). The recent identification of aurintricarboxylic acid (ATA) as a strong inhibitor of PMCA4 (Mohamed *et al.,* 2013) *prompted us to hypothesise that inhibition of PMCA4 with ATA will enhance VEGF-induced angiogenesis.*

To test this hypothesis we have established the following aims for this thesis:

1) To determine the activity of the calcineurin/NFAT pathway in endothelial cells when PMCA4 activity is inhibited by treatment with ATA.

2) To determine the effect of PMCA4 inhibition by ATA on the expression of VEGF-responsive, pro-angiogenic, NFAT-target genes.

3) To examine the effect of ATA-mediated inhibition of PMCA4 on the interaction between PMCA4 and calcineurin in endothelial cells.

4) To investigate the effect of PMCA4 inhibition by ATA on endothelial cell motility.

5) To determine the effect of ATA-mediated inhibition of PMCA4 on endothelial cell tubular morphogenesis.

6) To examine the effect of ATA on the viability of endothelial cells.

7) To determine the effect of ATA on the interaction of PMCA4 with endothelial nitric oxide synthase (eNOS).

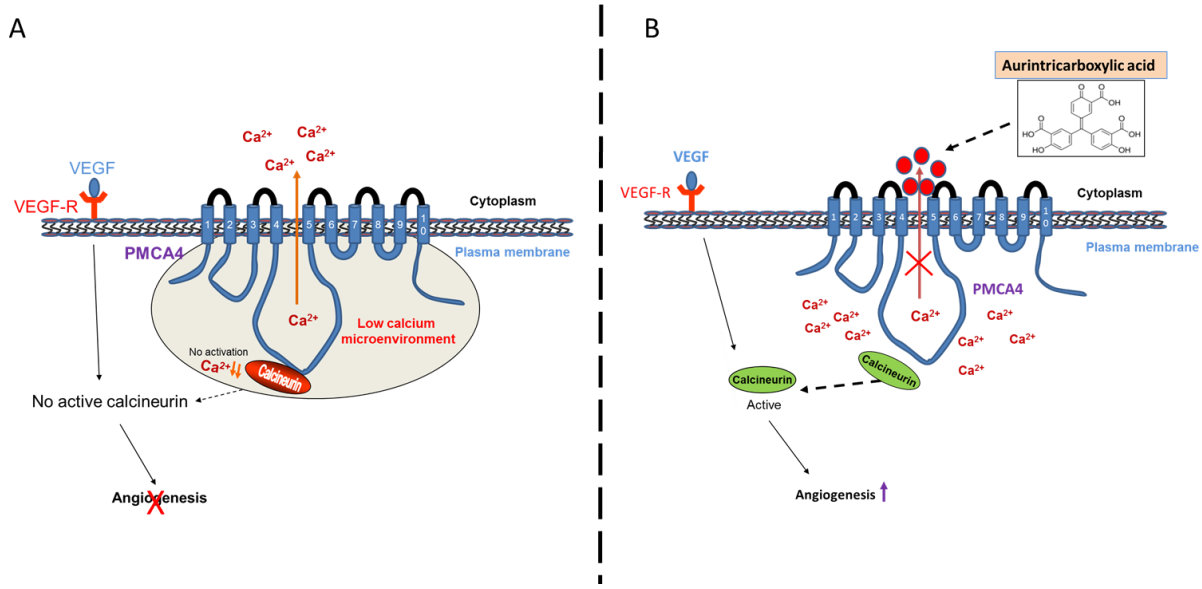


Figure 2.1 Hypothesis tested in this study. Inhibition of PMCA4 with ATA enhances VEGF-induced angiogenesis. A) Interaction of PMCA4 with calcineurin leads to downregulation of calcineurin activity and, in doing so, attenuates calcineurin-mediated angiogenesis in response to VEGF. B) Inhibition of PMCA4 with ATA reverses the negative effect exerted by PMCA4 on calcineurin, which can be fully activated in response to VEGF stimulation leading to an improvement in the associated pro-angionenic processes.

CHAPTER 3

Materials and Methods

3.1 Tissue culture

Cell culture procedures were carried out under aseptic conditions using a class II microbiological safety cabinet (BioMAT 2, CAS, UK) sterilised with 70% ethanol (Sigma-Aldrich, UK) and 1% Trigene (Sigma-Aldrich, UK) solution, to prevent the risk of contamination of cells. All solutions required for tissue culture were warmed to room temperature prior to use. Cells in culture were routinely passaged to maintain and amplify cells. To do this, following aspiration of the medium from the culture flask, cells were washed with 1x phosphate buffered saline (PBS 1x) and detached with a 0.25% trypsin-EDTA solution. Cells were checked under the microscope to ensure they were detached, and fresh complete media was added to neutralize the trypsin. The contents of the flask were transferred into a sterile universal tube and centrifuged at 1200 rpm for 5 minutes. After removing the supernatant, pelleted cells were re-suspended in fresh medium and transferred to tissue culture flasks of required size. Cells were then incubated at 37ºC, 5% CO_2.

3.1.1 Recovering cells from liquid nitrogen

A cryovial with 3 x 10^6 cells frozen in a solution of 10% dimethyl sulfoxide (DMSO) in fetal bovine serum (FBS) was carefully removed from the liquid nitrogen storage tank and thawed at 37ºC. The cell solution was diluted 1:10 in tissue culture medium to prevent damage to cells and centrifuged at 1200 rpm for 5 minutes. The supernatant was carefully removed. The cell pellet was re-suspended in fresh tissue culture medium and transferred to T175 tissue culture flask. Cells were then cultured at 37ºC, 5% CO_2.

3.1.2 Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs purchased from TCS Cellworks were grown in tissue culture flasks pre-coated with 0.1% gelatin in Endothelial Cell Growth Medium (ECGM, PromoCell) supplemented with 1% penicillin/streptomycin/amphotericin B (Sigma-Aldrich) and ECGM-supplement mix (Promocell, UK) containing: 2% FBS, 0.4% endothelial cell growth supplement, 0.1

ng/ml epidermal growth factor (recombinant human), 1 ng/ml basic fibroblast growth factor (recombinant human), 90 µg/ml heparin, and 1 µg/ml hydrocortisone. HUVECs between passages 5 and 8 were used for experiments.

3.1.3 Human Embryonic Kidney 293A Cells (HEK293A)

HEK293A cells were cultured in tissue culture flasks pre-coated with 0.1% gelatin in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK) supplemented with; 10% FBS, 1.73 mM L-glutamine, 1% penicillin/streptomycin/amphotericin B (Sigma-Aldrich, UK). HEK293A were used for amplification of adenoviral vectors (see section 3.3.2).

3.1.4 Mouse Lung Endothelial Cells (MLECs)

Purified MLECs were plated on tissue culture plates pre-coated with 0.1% gelatine and cultured in MLEC medium consisting of DMEM/F-12 medium (Sigma-Aldrich, UK) supplemented with 20% FBS, 0.1 mg/ml heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich, UK), 4mM L-glutamine, 250 µg/ml endothelial cell growth supplement (AbD serotec, UK), and 1% penicillin/streptomycin/amphotericin B (Sigma-Aldrich, UK). MLECs were used at very early stages (either when freshly isolated cells reached 80% confluency or after one passage).

3.1.4.1 Isolation of Mouse Lung Endothelial Cells (MLECs)

Lungs removed from 6-8 week old wild-type (*PMCA4*$^{+/+}$) and knockout (*PMCA4*$^{-/-}$) mice (Schuh *et al*., 2004) were a generous gift from Dr Elizabeth Cartwright (Cardiovascular Research Group, University of Manchester, Manchester, United Kingdom). Lung samples were first transferred to DMEM-F12 serum-free medium, and then washed in 70% ethanol for 5 seconds. Traces of ethanol were removed by placing lungs in MLEC medium for 30 seconds. Lungs were then minced into a paste, and digested in 10 ml of a 440 units/ml solution of Type I collagenase (Gibco, UK) for exactly one hour at 37ºC. Freshly prepared collagenase was used for each round of MLEC isolation. To prepare

collagenase digestion solution, 0.1 g Type I collagenase was added to 25 ml of PBS 1x. The mixture was incubated at 37ºC for 1 hour to maximise solubility of collagenase, and then 25 ml more of PBS were added to generate a final concentration of collagenase 440 units/ml. The solution was sterilised by filtration and pre-warmed at 37ºC before use. Lung digestion was stopped by adding 10 ml of MLEC medium and then the tissue was disrupted by passing everything through a 19 gauge needle four times. Remaining chunks of lungs were removed by filtering the solution through a 70 µm cell strainer. The filtrate was added to a tube containing 20 ml of MLEC medium. Lung cells were collected by centrifugation for 10 minutes at 1200 rpm. The supernatant was removed carefully, and the pellet re-suspended in 10 ml of MLEC medium and plated onto Primaria™ 100 mm tissue culture dishes (BD biosciences, UK) that had been pre-coated for at least two hours with 0.1% gelatin and 0.1 mg/ml fibronectin from bovine plasma (Sigma-Aldrich, UK). Cells were incubated at 37°C, 5% CO_2 overnight. The following day, the mixed population of cells was subjected to negative selection in order to remove macrophages and other immune cells that display the FcγIII/II receptor. For this purpose, the medium was aspirated and cells were washed three times with PBS 1x to completely remove cells in suspension. Then 5 ml of fresh MLEC medium were added and cells were incubated at 4°C for 20 minutes. After incubation, MLEC medium was replaced with 3 ml of a 1.7 µg/ml solution of purified rat anti-CD16/CD32 (FcγIII/II receptor) antibody (BD Biosciences, UK) in PBS 1x, and cells were further incubated at 4°C for 30 minutes with regular rotation of the plate, to ensure that the whole surface of the plate was covered with antibody solution at all times. The antibody solution was removed after incubation. Cells were washed once with PBS 1x, and 3 ml of magnetic beads conjugated anti-rat IgG antibody solution (Dynabeads, Invitrogen) ($6.7x10^6$ beads/ml in MLEC medium) were added to the plate that was incubated at 4°C for 30 more minutes with gentle agitation. Cells were washed three times with PBS 1x,

detached with trypsin and re-suspended in 15 ml of MLEC medium. The solution was transferred to a centrifuge tube that was then placed in a magnetic holder for 7 minutes to direct macrophages attached to magnetic beads to the wall of the tube that was in contact with the magnet. Cells in suspension were carefully pipetted out of the tube while avoiding to taking any beads, and placed into a pre-coated 10 mm tissue culture dish. Plates were incubated at 37ºC, 5%CO_2. MLEC medium was changed every day until colonies of approximately 20-30 cells were visible under the microscope. The cells were cultured normally for 5-7 days. The variability of incubation time is required in different isolation experiments was probably due to differential digestion with Type I collagenase, or minimal variations in the strength applied when the tissue was disaggregated by passing through a 19-gauge needle. Also, minor differences might be caused by small variation in the length of time between harvesting lungs and platting cells, or small differences in age of the mice. No difference in MLEC recovery were observed when using mice of different gender. Endothelial cells were then selected using an antibody directed against the endothelial cell marker ICAM-2 (CD102). The same procedure described above for negative selection was performed in this step, except that a purified rat anti-CD102 antibody (BD Biosciences, UK) was used to select the cells, and that in this case, cells in suspension were discarded whereas magnetic beads with attached endothelial cells were recovered, resuspended in 10 ml of MLEC medium and plated in pre-coated 100 mm tissue culture dishes. Cells were incubated at 37ºC, 5% CO2 and medium replaced every day until cells reached confluency. The purity of the culture in endothelial cells was monitored using flow cytometry by determining the percentage of cells in the population expressing the endothelial cell marker ICAM-2 (CD102).

Deficiency of PMCA4 in cells isolated from *PMCA4*$^{-/-}$ mice was checked by qRT-PCR and western blot.

3.1.5 Maintenance of cell cultures

Cells were routinely monitored for any changes in the growth medium and cell population. The medium was changed every 1-2 days or when there was a change in the colour of the medium indicating a drop in pH. Cells were incubated at 37ºC, 5% CO_2 until reaching confluency.

3.1.6 Counting cells

To determine the number of cells in a cell suspension, an aliquot of 10 µl of mixed cell suspension was placed in a haemocytometer. Cells were counted under the microscope. The number of cells in 1 ml of the suspension is calculated by multiplying the number of cells counted in the haemocytometer x 10^4.

3.1.7 Freezing cells

Following trypsinisation, the cell suspension (3 x 10^6 cells) was centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 1 ml of freezing medium (90% FBS, 10% DMSO), transferred to a labelled cryovial (Nalgene Cryoware™ Labware, Roskilde, Denmark) and stored at -80ºC for 2 days to ensure slow freezing before transferring vials to a liquid nitrogen storage tank (-196ºC). DMSO was used in the freezing medium as a cryoprotective agent. Initial freezing at -80ºC for 2 days with subsequent transfer to liquid nitrogen guaranteed a slow cooling process that reduces the risk of ice crystal formation and diminishes cell damage during the freezing process.

3.2 Cell stimulation

Where specified, some experiments required cell stimulation. To do this cells were washed with PBS 1x to completely remove any remaining medium containing serum and cells were then incubated in the corresponding serum-free medium with 0-2% FBS without any supplementary growth factors for the required time.

HUVECs and MLECs were stimulated with VEGF 25 ng/ml (Peprotech). To prepare VEGF solution, 10 µg lyophilized form of VEGF was dissolved in 400 µl of PBS 1x. 5 µl of this stock solution were added to 5 ml of culture medium to make a final concentration of VEGF 25 ng/ml.

HUVECs were stimulated with bFGF 50 ng/ml (Peprotech). To prepare this stock solution, 10 µg of bFGF was added to PBS 1x (400 µl). 10ul of stock bFGF solution were added to 5 ml of culture medium to make a final concentration of bFGF 50 ng/ml.

HUVECs and MLECs were treated with ATA at 250 nM. To prepare an ATA stock solution, 4 mg of ATA powder (Sigma-Aldrich, UK) were added to 10 ml of DMSO. The mixture was mixed thoroughly, and then diluted 1:5 in DMSO. 6.25 ul of this stock solution were added to 5 ml of the corresponding cell culture medium to generate a final concentration of 250 nM.

3.3 Transfection of endothelial cells using replication-deficient adenoviral vectors

3.3.1 Adenoviruses used in this study

Viruses (Ad-NFAT-Luc and Ad-LacZ) used for this study were kindly provided by Dr Delvac Oceandy and Dr Elizabeth J. Cartwright from the Division of Cardiovascular Sciences, University of Manchester, Manchester Academic Health Sciences Centre, Manchester, UK. Replication-deficient adenovirus Ad-NFAT-Luc harbours an NFAT-dependent, luciferase-based reporter vector encoding luciferase. The expression of the luciferase gene in this vector is controlled by 3 NFAT-binding motifs placed upstream of a minimal promoter region. Generation of this vector has been previously described (Baggott *et al*., 2014). This vector was used to monitor the activity of the calcineurin/NFAT signalling pathway in endothelial cells. Replication-deficient adenovirus Ad-LacZ encodes the β-galactosidase protein and was used as a control (Baggott *et al*., 2014).

Replication-deficient adenovirus Ad-ID4 encodes a Flag-tagged version of the fragment encompassing aminoacids 428 to 651 of human PMCA4 that corresponds to the domain implicated in the interaction with calcineurin (Buch *et al*., 2005). This vector was generated in our laboratory and its generation has been previously described (Baggott *et al*., 2014). This adenovirus was used to express the polypeptide Flag-PMCA4(428-651) in endothelial cells to disrupt the interaction between endogenous PMCA4 and calcineurin as it has been reported (Baggott *et al.,* 2014).

A schematic representation of the features of these 3 vectors is shown in Figure 3.1.

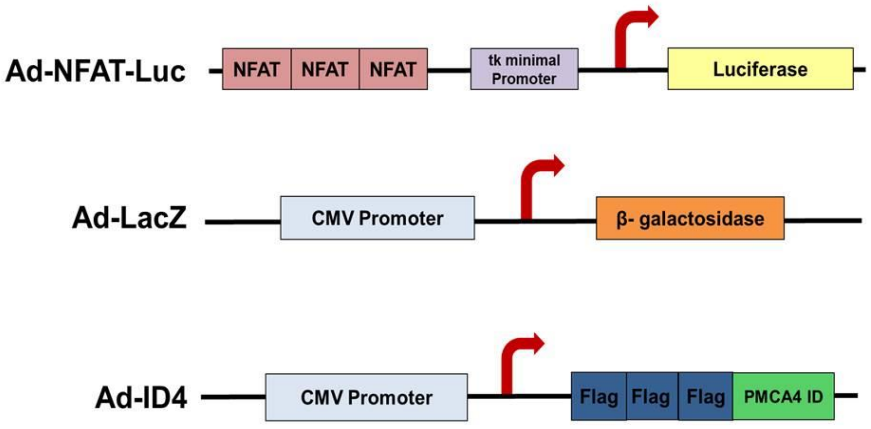


Fig 3.1 Main features of the adenoviruses used in this study. Ad-NFAT-Luc is an NFAT-dependent adenovirus encoding the protein Luciferase. Luciferase expression is driven by NFAT ocupancy of three NFAT binding sites placed in tandem upstream of the minimal promoter of the *timidine kinase* (tk) gene. In Ad-LacZ, the expression of β-galactosidase is controlled by the promoter of cytomegalovirus (CMV). Ad-ID4 encodes a Flag-tagged version of the region 428-651 of human PMCA4b that corresponds to the domain of PMCA4b that interacts with calcineurin. Expression of Flag-PMCA4(428-651) in this vector is driven by the CMV promoter.

3.3.2 Amplification of adenoviruses

Low passage number HEK293A cells were cultured in T175 tissue culture flasks as described. Secondary stock of adenovirus was generated by infection of HEK293A cells with a primary stock of virus (50 µl/flask) for 3-5 days until all cells showed symptoms of

the cytotoxic effect of adenovirus infection (cells become rounded and detached). The virus was collected by transferring cells/medium to falcon tubes. Medium with cells was centrifuged at 1500 rpm for 5 minutes at room temperature. The supernatant was discarded with great care and the pellet was washed in 5 ml of PBS 1x. Cells were re-centrifuged as above and the pellet finally re-suspended in 0.5 ml/flask of PBS 1x. Virus was stored at -80ºC or processed for further amplification.

Tertiary stock of the virus was produced by infection of confluent HEK293A cells with a secondary stock of virus generated in the previous step (50 µl/flask). Cells were incubated with virus until all cells had become rounded and detached. Cells containing virus were collected by centrifugation, washed with PBS 1x as described above, and resuspended in 0.5 ml/flask of PBS. Purification of the virus was performed by adding an equivalent volume of chloroform. The solution was mixed continuously by inversion for 5 minutes and then centrifuged for 15 minutes at 3000 rpm at room temperature. The top phase (containing the virus) was collected with extreme care and stored at -80°. To determine the number of adenovirus particles in the tertiary stock of the virus, HEK293A cells were seeded in at 5 x 10^3 cells/well in 96-well tissue culture plates, and incubate overnight at 37ºC. The following day the medium was removed and replaced with 100 µl of serially diluted adenovirus stocks (diluted in DMEM medium), and cells incubated for 24 hours. Each dilution point was analysed in triplicate. After this period, medium containing virus was removed and 100 µl of fresh DMEM was added to each well. The medium was changed on day 4 and plaque formation was monitored each day. On day 8 following infection final dilution which shows plaque formation were taken as the endpoint from the assay. Appendix 4 shows detailed correlation of virus dilution and number of infectious adenovirus particle (Pickard 2007).

3.3.3 Adenoviral Infection

To determine the activity of the calcineurin/NFAT pathway, HUVECs were cultured in 6-well tissue culture plates at a density of 3 x 10^5 cells/well, and then infected with Ad-NFAT-Luc at a MOI=50, for 48 hours at 37°C, 5% CO_2. After this period, medium containing virus was removed, cells were washed with PBS 1x twice, and then incubated overnight in ECGM basal medium supplemented with 0.5% FBS for serum-starvation of the cells. The following morning cells were stimulated as indicated and luciferase activity determined.

To disrupt the interaction between PMCA4 and calcineurin, HUVECs seeded in T-75 tissue culture flasks at a density of 2.5 x 10^6 cells/flask were infected with Ad-LacZ or Ad-ID4 at a MOI=150 for 72 hours, and used for migration and western blot assays as described in later sections.

3.4 Protein Determination Assays

3.4.1 Collection of total proteins

Total proteins were collected from whole cells by washing the cells with PBS 1x and directly lysing the cells in NuPAGE® LDS sample buffer (Life Technology, UK) containing 0.05% beta-Mercaptoethanol. As a reference, cells growing in a 6 well-plate were lysed in 100 µl of PAGE-LDS buffer per well. Cell lysate was heated at 100°C for 10 minutes to solubilise DNA and then cooled down on ice before storing at -20°C until used.

3.4.2 Isolation of plasma membrane-associated proteins

To determine the interaction between PMCA4 and calcineurin proteins in the plasma membrane, HUVECs were seeded at a density of 4 x 10^6 cells/flask in a T-75 tissue culture flask and incubated at 37°C, 5% CO_2. The following day, cells were serum-starved for 3 hours in ECGM basal medium (Promocell) without serum, and stimulated with VEGF (25 ng/ml) in the presence or absence of 250 nM aurintricarboxlic acid (ATA) for 4 hours. To isolate membrane-associated proteins we used a ProteoExtract

Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's instruction. Briefly, medium was carefully removed and cells washed with 5 ml of ice-cold wash buffer with gentle agitation at 4°C for 5 minutes. After washing, the buffer was completely removed. Ice-cold extraction buffer I was prepared by mixing 2 ml of extraction buffer with 5 µl of protease inhibitor cocktail, and added into the flasks. It was ensured that the buffer covered all the surface of the flask, and cells were incubated for 10 minutes with gentle agitation at 4°C. This buffer extracted only cytoplasmic proteins as indicated by the manufacture. Lysate containing cytoplasmic proteins was discarded. Ice-cold extraction buffer II was prepared by mixing 2 ml extraction buffer II with 5 µl protease inhibitor cocktail, and added into the flasks making sure that all the surface of the flask was covered with buffer. Cells were incubated in buffer II for 30 minutes at 4°C with gentle agitation. This buffer extracted membrane-associated proteins. The buffer containing membrane-associated protein was recovered and spun at 13000 rpm for 2 minutes to clear debris. Protein sample was transferred to an Amicon Ultra-0.5 centrifugal filter device, and concentrated by centrifugation at 14000 rpm for 30 minutes at 4°C. Protein concentration in the samples was measured using a BCA protein assay, and equal amount of proteins were analysed by western blot.

3.4.3 Protein concentration measurement

To determine the concentration of proteins in protein lysates we used a BCA^{TM} Protein Assay Kit (Thermo Scientific) according to the manufacturer's recommendations. Briefly, serial dilutions of Bovine Serum Albumin (BSA) protein were freshly prepared for each assay and used as a reference. The assay was performed by preparing a 50:1 mixture of Pierce BCA reagent mix A and B respectively. 200 µl of Reagent A:B mixture were added to 25 µl of sample (or BSA standard) and incubated at 37°C for 20 minutes in the dark. Absorbance of samples was read at 540nm using a multiwell plate reader

spectrophotometer (Thermo LabSystems) and protein concentration of the samples was calculated using the reading of BSA standard samples as a reference.

3.5 Immunoprecipitation (IP)

To determine the interaction of PMCA4 protein with calcineurin and eNOS in VEGF-stimulated HUVECs, we carried out immunoprecipitation assays using a Pierce™ Co-Immunoprecipitation kit (Sigma-Aldrich, UK). HUVECs were cultured in T175 tissue culture flasks at 37°C, 5% CO_2 until reaching confluency. Confluent cells were serum-starved in ECGM medium without any serum for 3 hours, and then stimulated with VEGF (25 ng/ml) in the presence or absence of ATA (250 nM). After treatment, cells were lysed using ice-cold IP Lysis/Wash buffer and the lysate was transferred to an eppendorf tube and kept at 4°C on ice until use. For immunoprecipitation of the PMCA4/calcineurin complex, 15 µl of anti-calcineurin A monoclonal antibody (Sigma-Aldrich, UK) was coupled to 100 µl of AminoLink plus coupling Resin (Pierce) by incubation in ‘’Coupling buffer 1x’’ (Pierce) with rotation for 120 minutes at room temperature. The resin-antibody complex was washed twice with ‘’Wash Buffer’’ provided in the kit, and then incubated with 4 mg of protein lysate overnight, at 4°C with rotation. The following day, the resin was extensively washed with ‘’Wash buffer’’ and finally, immunoprecipitated proteins were eluted with 60 µl of ‘’Elution Buffer". 20 µl of 4x NUPAGE sample buffer containing 0.05% beta-mercaptoethanol were added to the samples.

For immunoprecipitation of the PMCA4/eNOS complex, the same procedure was followed except that 10 µl of the anti-PMCA 5F10 monoclonal antibody (Abcam) was used for immunoprecipitation.

3.6 Western Blot

3.6.1 Protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

A mixture of proteins was separated according to size by SDS-PAGE using a standard twin-plate mini gel unit system (SCIE-PLAS). A resolving gel solution with a percentage of acrylamide/bis-acrylamide between 6% and 12% was prepared depending on the size of the protein to be detected. This was added first to a gel casting device between two glass plates. After polymerisation, a stacking gel solution was placed on top of the resolving gel and a comb was immediately inserted on the top of the stacking gel solution to form wells required for loading of protein samples. A detailed protocol describing the reagents and amounts used in the preparation of the gel solutions has been included in ‘’Appendix 1’’ at the end of this thesis.

Protein samples were heated at 100°C for 2 minutes, and cooled down on ice very briefly before loading. 10 µl of BLUeye protein ladder (GeneFlow, UK) or 10 µl of Protomarkers^{TM} (National diagnostics) pre-stained protein ladder were loaded as markers of protein molecular weight.

Gels were run in Tris-Glycine buffer (0.025M Tris, 0.192M Glycine, 0.1% SDS) at 200V until the mixture of protein markers was clearly separated.

3.6.2 Protein blotting to PVDF membrane

Proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane using a wet-transfer blotting apparatus (Invitrogen) according to the following procedure. Prior to set up of the blotting transfer equipment, a PVDF membrane of the size of the PAGE resolving gel was hydrated with pure methanol and subsequently washed in dH_2O to remove methanol traces. Protein transfer was carried out by placing the gel and membrane between a ‘‘sandwich'' formed by several layers of Whatman 3 MM chromatography paper and foam sponges, both soaked in transfer buffer (0.025M Tris, 0.192M Glycine, 20% methanol).

Protein transfer was carried out at 35V for 90 minutes with all the components immersed in transfer buffer. After completion of the process, the membrane was

separated from the gel and successful transfer was verified by visualisation of the pre-stained protein markers in the membrane.

3.6.3 Protein Detection

After protein transfer, membranes were incubated in a 5% solution of skimmed milk in Tris Buffered Saline (TBS) 1x for 1 hour with agitation, in order to prevent non-specific binding of primary and secondary antibodies to the membrane. After completing this blocking step, the milk solution was removed and the membrane briefly washed with TBS-T (TBS-0.05% Tween 20). Appendix 2 shows a detailed description of the primary antibodies and conditions used in this study. For detection of Flag-tagged proteins, the membrane was incubated with a 1:2000 solution of anti-Flag M2 monoclonal HRP conjugated antibody (Sigma-Aldrich, UK) for 3 hours, at room temperature, washed in TBS-T five times for 5 minutes each, and developed following the ECL method described below.

In all other cases, membranes were incubated overnight, at 4°C, with agitation, in a solution of a primary antibody recognising specifically the protein to be detected diluted in TBS-T (Appendix 3). The following day, the solution containing primary antibody was removed, and membranes washed five times with TBS-T for 5 minutes each. Primary antibodies bound to the protein were detected by incubation for 3 hours at room temperature with a solution of a HRP-conjugated secondary antibody recognising specifically IgGs from the host used to produce the primary antibody. Unbound secondary antibody was removed by washing the membrane five times for 5 minutes each with TBS-T.

Finally, membranes were developed using an EZ-ECL chemiluminescence detection kit (GeneFlow, UK) by mixing ‘’Solution A’’ and ‘’Solution B’’ from the kit at a 1:1 ratio, and incubating the membrane in the resulting solution for 1 minute. Signal was detected by

exposing the membrane to Kodak BioMax MS auto-radiographic film (Sigma-Aldrich, UK) in dark conditions for different periods of time and subsequent development.

Protein levels were quantified by digital scanning of films and detection of band intensity using ImageJ software (National Institute of Health, USA).

3.7 Quantification of RNA gene expression

3.7.1 RNA isolation and purification

Quantitative real time-PCR analysis was used to determine the successful knockout of PMCA4 in isolated MLECs. *PMCA4*$^{+/+}$ and *PMCA4*$^{-/-}$ cells were plated at a density of 4 x 10^5 cells/well in pre-coated 6-well tissue culture plates and incubated at 37°C, 5% CO2 for 24 hours. The medium was removed and cells washed in PBS 1x. Total RNA was isolated using a RNA purification kit (Norgen, Canada). Cells were lysed using 300 µl of buffer RL and lysate stored at -80°C or processed immediately for RNA isolation. Absolute ethanol (100%) was added to the lysate and the mixture vortexed for 10 seconds at room temperature. Then the solution was passed through spin-columns by centrifugation at 13000 rpm for 60 seconds. 100 µl of a DNase I solution (Norgen, Canada) were added to the columns and incubated for 15 minutes to remove any DNA present in the sample. After this time, the DNAse I solution was removed by centrifugation and the columns thoroughly washed with wash solution. RNA was eluted by addition of 50 µl of RNA elution buffer directly to the dry columns and incubation for 2 minutes at room temperature. Purified total RNA was then collected by centrifugation at room temperature for 2 minutes at 2000 rpm, followed by centrifugation for 1 minute at 13000 rpm.

3.7.2 RNA Quantification

RNA concentration and quality of each sample was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK). The ratio of absorbance at 260nm and 280nm was used to assess the purity of RNA in the sample.

3.7.3 Complementary DNA (cDNA) synthesis

A high capacity cDNA reverse transcription kit (Applied Biosystems, UK) was used to synthesise cDNA from total RNA isolated in section 3.7.1. After RNA quantification, samples were diluted to 500 ng of total RNA in a final volume of 10 µl with nuclease-free water (Promega, UK). 10 µl of total RNA solution (50 ng/µl) were mixed with 10 µl of a 2x Reverse Transcription mixture containing 2x Retro Transcription buffer, 8mM dNTP mix, 2x random primers, and RNAse inhibitor (2 units/µl). This 2x RT master mix was mixed with each sample to make a final volume of 20 µl. Reverse transcription was carried out in a thermal cycler (PTC-200 Peltier Thermal Cycler, MJ Research) using the following conditions; 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes. After this time, the cDNA was placed on ice for immediate use or stored at -20ºC for long term storage.

3.8. Quantitative real-time PCR (qRT-PCR)

The quantification of gene-specific mRNA expression was determined by qRT-PCR using TaqMan Gene Expression Assays (Thermo Scientific, UK) with FAM reporter. The assay includes a pre-designed primer-pair and a unique probe for each target under investigation. For each test, the cDNA reaction was diluted 1:4 with nuclease-free water and 5.6 µl of this dilution added to a reaction mix consisting of 10 µl of a 2x realtime qRT-PCR master mix (Thermo Fisher), 1 µl of TaqMan Gene Expression assay-and 3.4 µl PCR grade nuclease-free water to make a final volume of 20 µl. A negative control sample that contained 5.6 µl of nuclease-free water instead of cDNA solution was included in the assay to verify the fidelity of the PCR amplification. The reactions were prepared in MicroAmp® fast optical 96-well reaction plates (Applied Biosystems, UK). After setting up the reaction, the plate was sealed with an optically clear seal, gently tapped to remove any air bubbles and placed on a 7500 Fast Real-Time PCR System (Applied Biosystems, UK). PCR cycling conditions consisted of an initial enzyme

activation step at 95ºC for 10 minutes, followed by denaturation at 95ºC for 15 seconds and annealing and extension at 60ºC for 1 minute for 40 cycles. Data were exported to GraphPad Prism for statistical analysis. The expression of the murine *GAPDH* gene was determined in parallel as control.

3.9 Luciferase reporter assay

To study the effect of ATA on the activity of the calcineurin/NFAT signalling pathway, primary HUVECs were seeded at a density of 3 x 10^5 cells/well in a 6-well tissue culture plate the day before viral infection. The following day, cells were infected with Ad-NFAT-Luc (an NFAT-dependent luciferase-based reporter vector) at a multiplicity of infection (MOI) = 50 and cells were incubated at 37ºC, 5% CO_2 for 48 hours. After infection, medium containing virus was removed and cells were serum-starved in ECGM medium containing 0.5% serum for 16 hours. The following day, cells were treated with ATA (250 nM) or DMSO for one hour and then stimulated with VEGF (25 ng/ml) for 6 hours. Stimulated cells were washed once with PBS and lysed in 60 µl of Cell Lysis Reagent 1x (Promega, UK) at room temperature for 10 minutes. Luciferase activity in the lysate was determined in a SIRIUS Luminometer V3.1 (Berthold detection systems, UK) by mixing 20 µl of the cell lysate and 100 µl of ‘’Luciferase Assay Substrate’’ from a Luciferase Assay System Kit (Promega, UK) and measuring the relative light units (RLU) produced after 30 seconds.

3.10 Wound healing migration assay

Cell migration assays of HUVECs and MLECs were carried out using the “Cytoselect™ 24-well wound healing assay kit” (Cell Biolabs). HUVECs were plated at a density of 7 x 10^5 cells/well in 0.1% gelatine pre-coated 24-well cell culture plates (COSTAR, UK), and a 0.9 mm gap was created using inserts from the assay kit. Cells were incubated for 24 hours at 37°C, 5% CO_2 for attachment. The following day, gap creators were removed and cells corresponding to time zero (t=0) were stained with a 0.6% crystal

violet/0.025% ammonium oxalate/5% ethyl alcohol solution (Sigma-Aldrich, UK) for 15 minutes at 37°C. Excess stain was removed by washing with PBS 1x three times and cells were fixed for 10 minutes at room temperature using formalin solution (10% neutral buffered containing formaldehyde 4% w/v) (Sigma-Aldrich, UK). Fixed cells were washed twice more with PBS1x and images were taken using a Nikon DSFi1 digital camera coupled to a Nikon ECLIPSE TS100 microscope at 4 x magnification. Gap creators were removed in the remaining wells and cells incubated in HUVEC medium supplemented with VEGF (25 ng/ml) in the presence of DMSO or 250 nM ATA for 24 hours (t=24). After incubation, cells were stained, fixed and images captured as above.

HUVECs Infected with Ad-LacZ and Ad-ID4 at MOI=150 were plated at a density of $7x10^5$ cells/well in pre-coated 24-well cell culture cluster plates (COSTAR®, UK). Migration assay was performed as described above.

Migration assay using *PMCA4*$^{+/+}$ and *PMCA4*$^{-/-}$ MLECs were carried out using the protocol mentioned above except that MLEC tissue culture medium was used in the assay, and VEGF was used at 50 ng/ml.

In all cases, the percentage of migration was quantified using ImageJ software (National Institute of Health, USA)

3.11 Matrigel tube formation assay

96-well tissue culture plates were pre-coated with Geltrex™ Matrix (low growth factors) (Invitrogen) and incubated at 37ºC for 30 minutes to allow the matrigel to solidify. HUVECs were detached from tissue culture flasks, and washed two times with Medium 200 without phenol red (Cascade Biologics™, UK) to remove any traces of serum and growth factors present in the ECGM culture medium. Cells were re-suspended in Medium 200 without phenol red and plated onto geltrex at a density of $1x10^5$ cells/well in Medium 200 containing 2% FBS and supplemented with growth factors (25 ng/ml VEGF or 50 ng/ml bFGF) in the presence of ATA or DMSO (vehicle) as a control. Plates

were then incubated for 16 hours at 37°C. After incubation, images were recorded using a Nikon DSFi1 digital camera coupled to a Nikon ECLIPSE TS100 microscope at 4x magnification. The percentage of tube formation was quantified by counting branching points from the recorded images.

3.12 MTT Assay

MTT assays were carried out to determine the cytotoxicity of ATA in endothelial cells. HUVECs were detached by trypsinisation, seeded on 96-well tissue culture plates (without gelatin pre-coating) at a density of $5x10^4$ cells/well, and incubated overnight at 37ºC. The following day one of the plates was analysed by MTT assay (t=0) by adding 50 µl/well of a 5 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) directly to the culture medium. Plates were wrapped in aluminium foil to keep the reaction in the dark and incubated for 4 hours at 37°C. After incubation, medium containing MTT solution was removed, and 175 µl/well of DMSO and 25µl of Sorensen's glycine buffer (0.1 M glycine-0.1 M NaCl equilibrated to pH10.5) were added to the cells. Absorbance at 540 nm was measured using a Multiskan Ascent plate reader (Thermo Labsystems, UK). In the remaining plates, the medium was replaced with HUVEC medium containing VEGF (25 ng/ml) and ATA or DMSO as required, and cells were further incubated at 37ºC, 5% CO_2 for 3 days before MTT analysis (T=3). In another set of plates (T=6) the medium was changed as above, cells incubated for 3 additional days, and viability assessed by MTT assay after the incubation period.

For incubation of endothelial cells with ATA for long periods, HUVECs were cultured in T75 tissue culture flasks and incubated for 7 days with vehicle (DMSO) or ATA in the presence of VEGF (25 ng/ml) prior to performing the assay.

3.13 Nitric Oxide determination

Intracellular nitric oxide (NO) bioavailability was determined using the NO-sensitive fluorescence probe DAF-FM (4-Amino-5-Methylamino-2',7'-Difluororescein Diacetate).

HUVECs were seeded in 12 mm Nunc bottom-glass dishes (Thermo Scientific) pre-coated with 0.1% gelatine at a density of 5 x 10^5 cells/plate, and incubated at 37ºC, 5% CO_2. The following day, cells were serum-starved by incubation in ECGM containing 0.5% FBS for 3 hours. After serum starvation, cells were incubated with L-Arginine (100 µM) for 15 minutes in the same serum-free medium. DAF-FM (Molecular Probes, UK) was added to the medium to a final concentration of 10 µm and cells incubated for 30 minutes. Medium containing dye was removed and replaced by basal ECGM with 0.5% FBS, and cells further incubated for 45 minutes at 37°C. After this period, cells were stimulated with VEGF (25 ng/ml) in the presence of ATA (250 nM) or vehicle (DMSO) for 5 minutes. After treatment, the reaction was stopped by removing the stimulus and washing cells with PBS 1x. Cells were fixed with Formalin (Sigma) for 15 minutes at room temperature, and then, washed three times in PBS 1X. Coverslips were mounted using mounting medium (Vector Laboratories, Inc). Fluorescence, as an indication of NO production, was detected using a ZEISS LSM 880 Confocal Laser Scanning Microscope.

3.14 Flow cytometry

Flow cytometry analysis was used to monitor the purity of endothelial cells obtained after MLEC isolation. Cells obtained from lungs isolated from *PMCA4*$^{+/+}$ or *PMCA4*$^{-/-}$ were cultured in 6 well plates pre-coated with 0.1% gelatin for 24 hours. The following day, cells were trypsinised and pelleted by centrifugation at 1200 rpm for 5 minutes. The supernatant was removed and the cells were washed with 5 ml of sterile PBS 1x twice. The final cell pellet was re-suspended in 200 µl of sterile PBS. Ice-cold methanol (500 µl) was slowly added to the cell suspension on a vortex mixer, and cells were fixed for 15 minutes on ice. After 15 minutes, the fixed cells were pelleted by centrifugation at 1200 rpm for 5 minutes, the methanol was removed and the cell pellet was washed with sterile 1x PBS. An antibody against ICAM-2 (CD102) was diluted 1:50 in FACS buffer

(0.1% BSA, 0.01% Triton X-100 (Sigma-Aldrich, UK), PBS) and this solution added to the cells for incubation at room temperature for 3 hours. After incubation, cells were washed with PBS1x to remove any unbound primary antibody and then incubated for 1 hour, at room temperature in the dark, with a FITC-conjugated secondary antibody prepared in FACS buffer at a dilution 1:500. After incubation, the cells were washed and re-suspended in FACS buffer and analysed using a Flow cytometer (BD Accuri C6).

CHAPTER 4

Results

4.1 (Aim 1) To determine the activity of the calcineurin/NFAT pathway in endothelial cells when PMCA4 activity is inhibited by treatment with ATA.

It has been previously reported by our group that PMCA4 negatively regulates the VEGF-mediated activation of the calcineurin/NFAT signal transduction pathway in endothelial cells (Baggott *et al.,* 2014). Additionally, the identification of the molecule aurintricarboxylic acid (ATA) as a potent inhibitor of isoform PMCA4 (Mohammed *et al.,* 2013) encouraged us to examine whether inhibition of PMCA4 with ATA improves VEGF-induced calcineurin/NFAT signalling. Thus our first aim was to determine whether inhibition of PMCA4 by ATA will affect the activity of calcineurin/NFAT pathway in endothelial cells stimulated with VEGF.

To test this possibility, HUVEC cells were infected with adenovirus Ad-NFAT-Luc (an NFAT-dependent, luciferase-based adenoviral reporter vector previously used by our group to determine the activity of the calcineurin/NFAT pathway in endothelial cells (Baggott *et al.,* 2104)). This vector contains 3 copies of the NFAT-binding site of the *IL-2* promoter placed upstream of the minimal promoter region of the thymidine kinase gene. The artificially created promoter is located upstream of the luciferase gene, and, therefore, expression of luciferase in the infected cells is highly dependent on activation of the calcineurin/NFAT pathway. Inhibition of PMCA4 by ATA reaches saturation at low concentrations of ATA in the range of 250 nM (Mohammed *et al.,* 2013). Therefore, in this study we decided to use this dose of ATA to achieve selective inhibition of PMCA4 while avoiding potential deleterious side-effects of the drug on the cells. Moreover, maintaining ATA at low concentration also ensured that the effects observed by treating cells with ATA are due to inhibition of PMCA4, and no of other molecules such as nucleases, calpain or chemokine receptors that have been reported to be inhibited by ATA at the 5-20 µM high concentration range (Posner *et al*., 1995; Hallick *et al*., 1997; Laufenberg *et al.,* 2014)). Infected cells were then treated with ATA (250 nM) or DMSO

for one hour and then stimulated with VEGF (25 ng/ml) for 6 hours after 18 hours serum starvation. Luciferase activity was then determined in these cells as a surrogate measurement of the activity of the calcineurin/NFAT pathway. As expected, stimulation of control cells with VEGF strongly induced the activity of the calcineurin/NFAT pathway (Figure 4.1). ATA treatment did not alter the activity of the calcineurin/NFAT pathway in control unstimulated cells, however, ATA further enhanced (78.1% increase) the strong activation of calcineurin/NFAT signalling induced by VEGF (Figure 4.1). This result supports our hypothesis that inhibition of PMCA4 function by ATA can reverse the negative effect exerted by the pump on calcineurin/NFAT signalling.

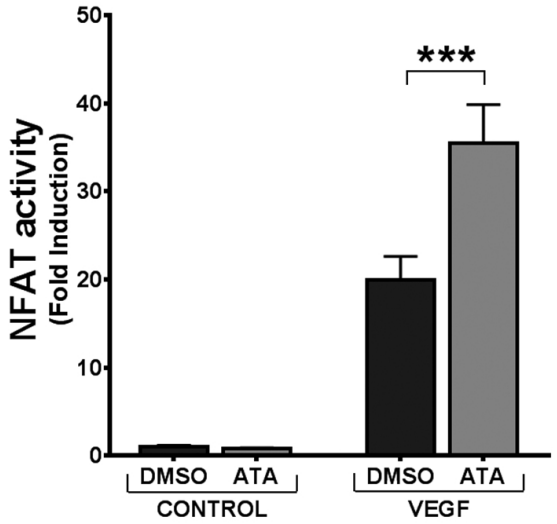


Figure 4.1 ATA enhances the VEGF-induced activation of calcineurin/NFAT signalling in endothelial cells. HUVECs infected with the NFAT-dependent, luciferase reporter adenovirus Ad-NFAT-Luc (MOI=50) were treated with VEGF (25 ng/ml), or left untreated (control), in the presence of ATA (250 nM) or vehicle (DMSO). Cells were lysed 6 hours post stimulation and luciferase activity determined as an indication of the activity of the calcineurin/NFAT pathway. Results are expressed as fold-induction over the calcineurin/NFAT activity detected in control cells treated with DMSO. Data are plotted as mean ± SE, n=12, obtained from 3 independent experiments. ***denotes statistically significant differences ($P \leq 0.005$, according to one-way ANOVA with *post hoc* Tukey´s comparison test) in the VEGF-induced NFAT activity of cells treated with ATA *vs* vehicle (DMSO).

4.2 (Aim 2) To determine the effect of PMCA4 inhibition by ATA on the expression of VEGF-responsive, pro-angiogenic, NFAT-target genes.

To investigate whether the increase in calcineurin/NFAT activity induced by ATA in cells stimulated with VEGF translates into an enhanced expression in VEGF-responsive, pro-angiogenic, NFAT-target genes we decided to analyse the expression of the protein RCAN1.4 in HUVEC cells stimulated under these conditions.

It has been widely demonstrated that VEGF strongly induces the expression of RCAN1.4 (also called DSCR-1) in a calcineurin/NFAT-dependent manner (Ryeom *et al.,* 2008; Holmes *et al.*, 2010; Minami *et al.,* 2009). The gene encoding RCAN1 is formed by 7 exons (Figure 4.2.1).

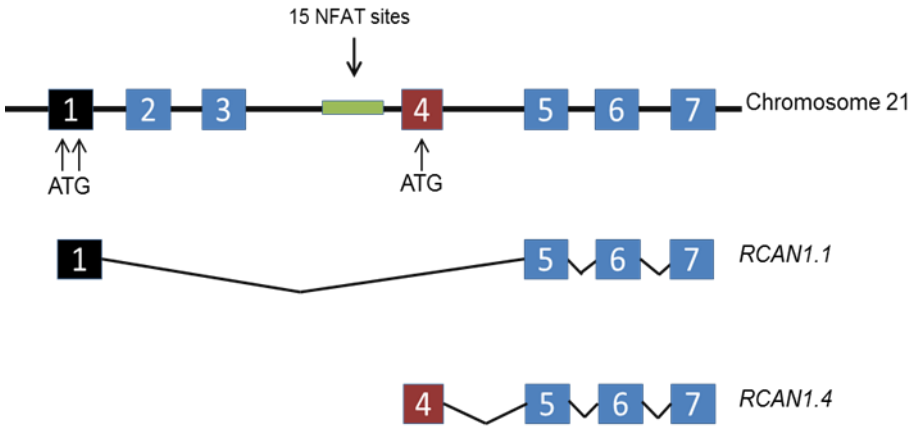


Figure 4.2.1 Genomic structure of the human *RCAN1* gene and alternative promoter usage (adapted from Holmes *et al.,* 2010). Exon structure of *RCAN1* gene illustrating alternative first exons in RCAN1.1 and RCAN1.4 isoforms. ATG indicates start codon for each isoform.

Exons 1-4 are alternative exons. Alternative promoter usage results in two different RCAN1 variants. Usage of the promoter region flanking exon 1 gives rise to variant RCAN1.1 that contains exons 1, 5, 6, and 7 (Figure 4.2.1). Usage of the genomic region flanking exon 4 give rise to variant RCAN1.4 that contains exons 4, 5, 6, and 7 (Figure 4.2.1). 15 NFAT-binding sites have been identified in the genomic region upstream of

exon 4 (Figure 4.2.1). Upon activation of the calcineurin/NFAT pathway by stimulation of endothelial cells with VEGF, occupancy of these NFAT-binding sites trigger strong up-regulation of RCAN1.4 expression. The strong dependence of this RCAN1 variant for activation of calcineurin/NFAT signalling prompted us to study the expression of this isoform in HUVECs stimulated with VEGF in the presence or absence of ATA.

For this purpose, HUVECs were serum-starved overnight in 0.5% fetal calf serum and then treated with ATA in the presence or absence of VEGF (25 ng/ml) for 4 hours. Protein lysates were analysed by western blot using an antibody against RCAN1.4 (Sigma-Aldrich, UK) (see appendix 2 for table of antibodies). In agreement with the increased calcineurin signalling triggered by ATA in VEGF-stimulated cells (Figure 4.1) ATA enhanced the expression of RCAN1.4 (56.8% increment) induced by VEGF in HUVEC cells (Figure 4.2.2).

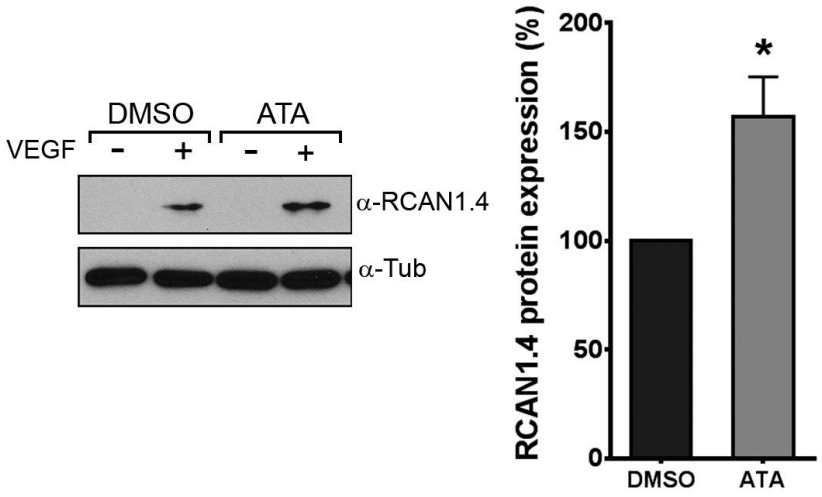


Figure 4.2.2 PMCA4 inhibition by ATA increases the expression of VEGF responsive, pro-angiogeneic, NFAT target protein RCAN1.4. HUVEC cells were serum-starved by overnight incubation in medium containing 0.5% fetal calf serum, and subsequently treated with VEGF (25 ng/ml), or left untreated as control, in the presence of ATA (250 nM) or vehicle (DMSO). Cells were lysed 4 hours post stimulation. Blots show representative images of the analysis of cellular protein lysates by western blot to determine the levels of RCAN1.4 and tubulin (loading control) in the samples. Band density was analysed using ImageJ software.RCAN1.4 levels were normalised according to the amount of tubulin in each sample. Results in histograms show data as mean ± SE, n=6, obtained from 3 independent experiments. *denotes statistically significant differences ($P \leq 0.05$, according to unpaired, two-tailed Student *t* test) when comparing cells treated with ATA *vs* vehicle (DMSO).

Concurrently with our results in section 4.1 ATA only increased RCAN1.4 expression in the presence of VEGF (Figure 4.2.2). A western blot detecting tubulin (Figure 4.2.2, lower panel) was carried out as a loading control to confirm equal loading in all samples. These data indicate that ATA potentiates the VEGF-induced activation of the calcineurin-NFAT pathway, and that this effect is translated into enhanced expression of NFAT-target genes such as *RCAN1.4.*

4.3 (Aim 3) To examine the effect of ATA-mediated inhibition of PMCA4 on the interaction between PMCA4 and calcineurin in endothelial cells.

It is thought that PMCA4 downregulates the activity of the calcineurin/NFAT pathway via a molecular interaction that sequesters calcineurin into low-calcium micro-domains created by the calcium extrusion activity of the pump (Holton *et al.,* 2010). Supporting this idea, we have demonstrated that disruption of the PMCA4/calcineurin interaction in endothelial cells abolishes the inhibitory effect of PMCA4 on calcineurin activity, leading to enhanced activation of this pathway (Baggott *et al.,* 2014). Therefore, we next assessed whether the enhancement in calcineurin signalling induced by ATA is associated to changes in the interaction between PMCA4 and calcineurin.

For this purpose, we used a ProteoExtract Subcellular Proteome Extraction Kit to isolate membrane-associated proteins from HUVECs treated with VEGF in the presence or absence of ATA, and analysed the contents of PMCA4 and calcineurin in this fraction. ATA treatment led to a significant reduction in the level of calcineurin associated to the plasma membrane, whereas the levels of PMCA4 remain unaffected (Figure 4.3.1A). Western blot analysis of the level of the plasma membrane protein cadherin was performed as a control to demonstrate equal amount of protein loading in all samples (Figure 4.3.1A).

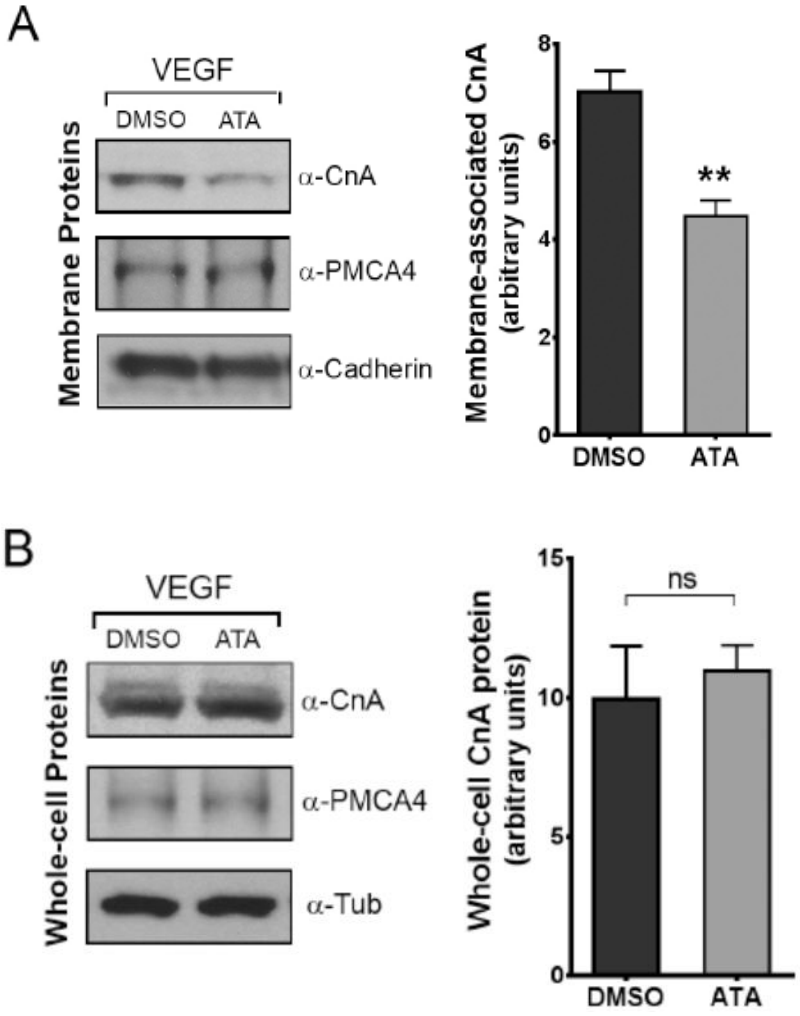


Figure 4.3.1 ATA treatment leads to a significant reduction in the levels of calcineurin present in the plasma membrane of VEGF-stimulated endothelial cells. HUVEC cells serum-starved by overnight incubation in medium containing 0.5% fetal calf serum were treated with VEGF (25 ng/ml) and ATA (250 nM) or vehicle (DMSO) for 4 hours. (A) Levels of calcineurin and PMCA4 present in the plasma membrane were determined by western blot analysis of the membrane-associated protein fraction with a 1:1000 dilution of an anti-calcineurin A antibody (α-CnA) or a 1:500 dilution of the JA3 anti-PMCA4 specific antibody (α-PMCA4). The level of Cadherin in the samples was analysed using a monoclonal anti-cadherin antibody (α-Cadherin) as membrane loading control. (B) HUVEC cells treated like described above were lysed in Laemmli buffer to isolate total proteins. Levels of calcineurin A (α-CnA), PMCA4 (α-PMCA4) and Tubulin (α -Tub) were determined by western blot using antibodies specific for these proteins. Histogram shows levels of total calcineurin normalised to the amount of tubulin present in each sample. In both figures (A,B), histograms show data as mean ± SE, obtained from 3 independent experiments. **indicates statistically significant differences ($P \leq 0.01$, according to unpaired, two-tailed Student *t* test) in the levels of calcineurin present in the membrane in VEGF-stimulated cells treated with ATA *vs* vehicle (DMSO). ns=non-significant.

To discard that reduction in membrane-associated calcineurin was attributable to downregulation of calcineurin expression in ATA-treated cells, we analysed the expression of this protein in total extracts isolated from whole cells. Total levels of calcineurin were equivalent in control (DMSO) or ATA-treated cells (Figure 4.3.1B). Likewise, analysis of total levels of PMCA4 in the same samples did not show any significant differences (Figure 4.3.1B). Equal protein loading in the sample was confirmed by western blot analysis of tubulin contents in the extracts of total protein used in the experiment (Figure 4.3.1B). These results suggest that treatment of HUVECs with ATA releases calcineurin from its interaction with PMCA4 in the plasma membrane.

To confirm that the decrease in the level of calcineurin associated to the membrane was the result of disruption of the PMCA4/calcineurin interaction, we performed immunoprecipitation experiments. Total protein extracts isolated from HUVECs stimulated with VEGF in the presence or absence of 250 nM ATA were immunoprecipitated with an anti-calcineurin antibody. Western blot of the immunoprecipitated proteins revealed that treatment with ATA significantly reduced the amount of PMCA4 co-precipitated with calcineurin, indicating dissociation of the PMCA4/calcineurin complex (Figure 4.3.2). Similar levels of calcineurin were precipitated in the presence or absence of ATA (Figure 4.3.2), ruling out the possibility that changes in the affinity for calcineurin of the immunoprecipitating antibody in ATA treated cells were the reason for the decrease in PMCA4 co-precipitation.

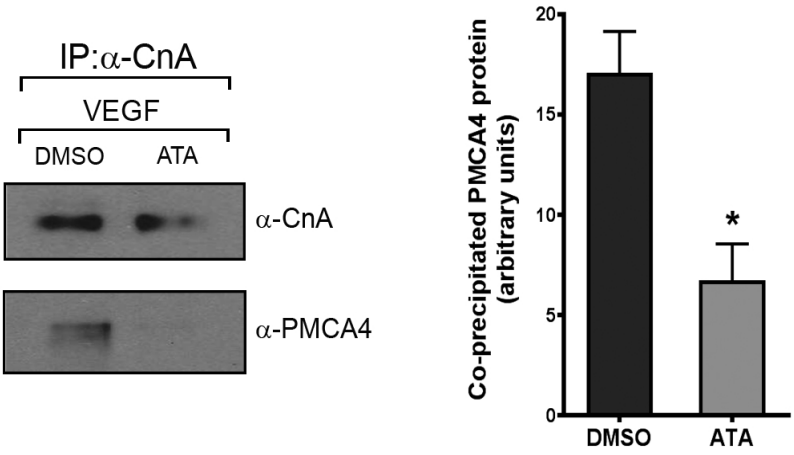


Figure 4.3.2 PMCA4 inhibition by ATA releases calcineurin from its interaction with PMCA4. HUVECs treated with VEGF (25 ng/ml) and ATA (250 nM) or vehicle (DMSO) for 4 hours, were lysed and total proteins immunoprecipitated with an anti-calcineurin antibody. Levels of calcineurin and PMCA4 in the precipitated proteins were determined by western blot (α-CnA and α-PMCA4, respectively). Histogram shows levels of co-precipitated PMCA4 normalised to the amount of calcineurin precipitated in each assay. Histogram represents mean ± SE, obtained from 3 independent experiments. *denotes statistically significant differences ($P \leq 0.05$, according to unpaired, two-tailed Student *t* test) in the amount of PMCA4 co-precipitated with calcineurin in VEGF-stimulated HUVECs treated with ATA *vs* vehicle (DMSO).

Altogether, the data presented in these sections suggest that ATA-treatment results in disruption of the PMCA4/calcineurin interaction. The release of calcineurin from the inhibitory effect exerted by PMCA4 would result in an enhancement of the VEGF-induced activation of the calcineurin/NFAT pathway that would lead to up-regulation in the expression of the endothelial NFAT-target gene *RCAN1.4.*

4.4 (Aim 4) To investigate the effect of PMCA4 inhibition by ATA on endothelial cell motility.

4.4.1 Effect of ATA on the motility of HUVEC cells

We have previously reported that PMCA4 downregulates endothelial cell migration and tubular morphogenesis *via* the establishment of an inhibitory molecular interaction with cytoplasmic calcineurin (Baggott *et al.,* 2014). The strong enhancement on the activity of the calcineurin/NFAT pathway induced by ATA in VEGF-stimulated HUVECs (Figure

4.1) prompted us to postulate that ATA should reverse the negative effect exerted by PMCA4 on these angiogenic processes. To investigate this possibility, HUVEC cells were plated in pre-coated 24-well cell culture plates and a 0.9 mm gap was created using a ‘’gap generator’’ from a wound healing assay kit (Cell Biolabs Inc). Cells were stained and fixed at time zero and after 24 hours of incubation with VEGF in the presence of absence of ATA. Images were taken at time zero and after 24 hours, and closure of the gap (indicating cellular migration) was analysed using ImageJ software. Endothelial cell migration was found to increase significantly (58.4 % increment) by ATA treatment compared to the migration of control cells treated with DMSO (Figure 4.4.1) highlighting that ATA increases HUVEC cell motility in response to VEGF.

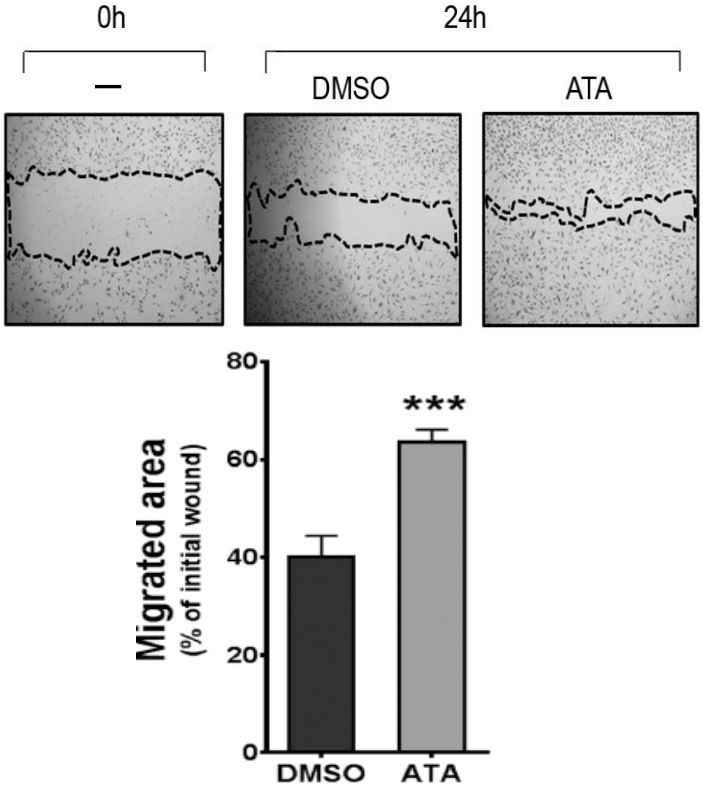


Figure 4.4.1 ATA treatment increases cell migration of HUVEC cells stimulated with VEGF. HUVEC cells plated on 24-well tissue culture plates to carry out wound healing migration assays, were fixed at time zero or after incubation in ECGM supplemented with VEGF (25 ng/ml) in the presence of ATA (250 nM) or DMSO for 24 hours. Images were taken using a Nikon DSFi1 digital camera coupled to a Nikon ECLIPSE TS100 microscope at 4x magnification and wound closure analysed with ImageJ software. VEGF-stimulated endothelial cell migration

was significantly increased by ATA treatment. Representative images of endothelial wound-healing migration assays are shown. Cell migration was determined by subtracting the value of the non-migrated area from the initial wound area (time=0), and expressing this value as a percentage of the total area at time zero. Histogram shows data as mean ± SE, n=9, obtained from 3 independent experiments. ***denotes statistically significant differences ($P \leq 0.005$, according to unpaired, two-tailed, Student *t* test) in the migration of cells treated with ATA *vs* vehicle (DMSO).

4.4.2 Analysis of involvement of PMCA4 on the ATA-mediated enhancement of endothelial cell motility

To determine that the improving effect of ATA on endothelial cell motility involves inhibition of PMCA4, and is not the consequence of an off-target effect in the cell, we performed migration assays using mouse lung endothelial cells (MLECs) isolated from *PMCA4*$^{-/-}$ knockout mice or their wild-type littermates.

To verify that our procedure for isolation of MLEC cells from mouse lungs yielded a population of endothelial cells without significant contamination by other pulmonary cell types, we analysed the percentage of cells in the isolated population expressing the endothelial cell marker ICAM-2 (CD102). Both *PMCA4+/+* and *PMCA4-/-* cells were used for flow cytometry analysis and showed that around 90% of the cells expressed ICAM-2 in the cell surface (Figure 4.4.2.1), confirming the enrichment of endothelial cells in the population after the isolation/purification procedure. No difference in the yield of isolated MLEC cells was observed when isolation was performed with lungs coming from *PMCA4+/+* and *PMCA4-/-* mice.

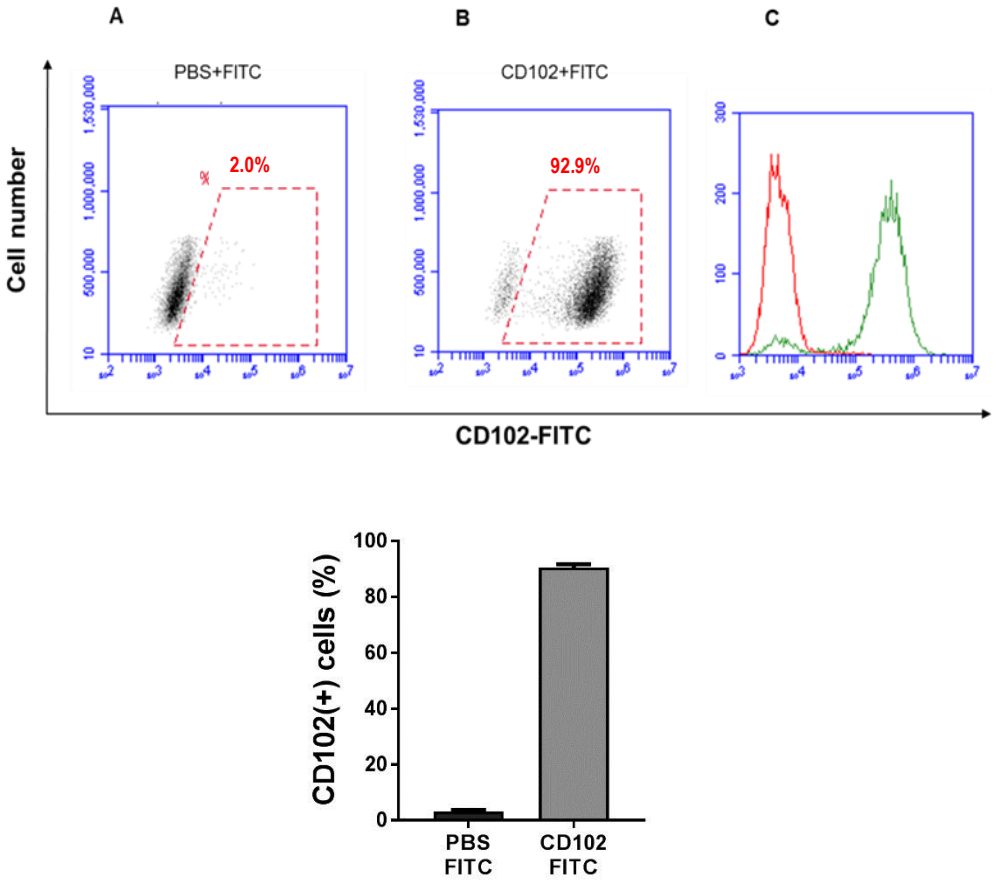


Figure 4.4.2.1 Flow cytometric analysis of endothelial cell marker (CD102) expression on MLEC cells. MLEC cells isolated from *PMCA4+/+* or *PMCA4-/-* mice were cultured in 6- well plates pre-coated with 0.1% gelatine for 24 hours. Expression of endothelial surface marker CD102 was measured by using flow cytometry analysis. A) Control flow cytometry analysis performed incubating the cell population only with anti-IgG FITC-conjugated secondary antibody. Most of the cells appear as CD102(-). (B) Flow cytometry analysis performed incubating the cells with primary anti-CD102 antibody and then with anti-IgG FITC-conjugated secondary antibody. 92.9% of the cells were detected as CD102(+). (C) Overlapping representation of CD102(+) cells in experiments (A) and (B). Fluorescence of control cells incubated only with anti-IgG(FITC) is shown in red. Fluorescence of cells incubated with anti-CD102 and anti-IgG(FITC) is shown in green. Images are shown above is from *PMCA4+/+* cells. Images are representative of three independent experiments. Histogram shows % of CD102(+) cells in flow cytometry analysis when the isolated cell population was stained as described in A (PBS+FITC, control) or in B (CD102+FITC). Data are shown as mean ± SE, n=7, obtained from 3 independent experiments of MLEC isolation/purification.

To demonstrate the successful knockout of *PMCA4* in the isolated MLEC cells, we collected RNA and protein samples from cells isolated from *PMCA4*$^{+/+}$ or *PMCA4*$^{-/-}$ mice and analysed the expression of the *PMCA4* gene in these cells by qPCR and western

blot, respectively. PMCA4 RNA and protein were clearly present in wild-type cells but neither were detected in cells obtained from knockout animals (Figure 4.4.2.2).

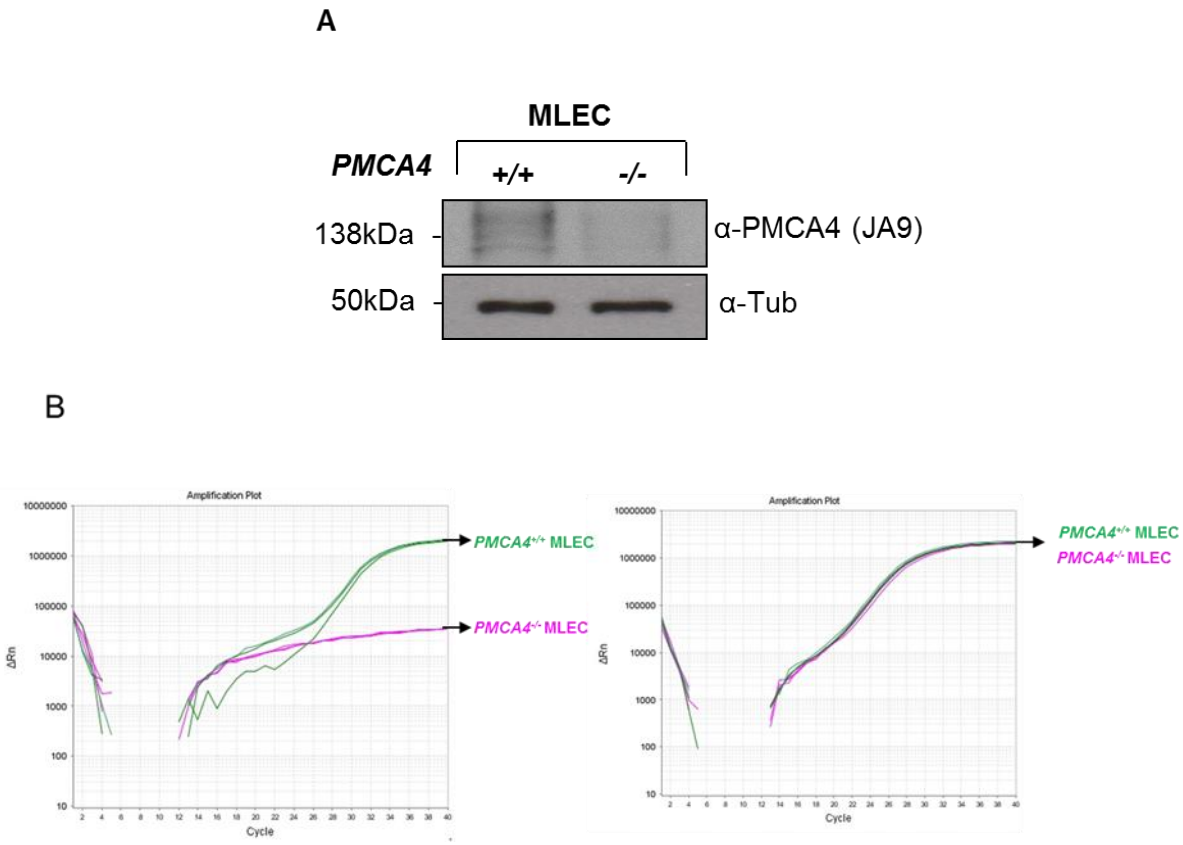
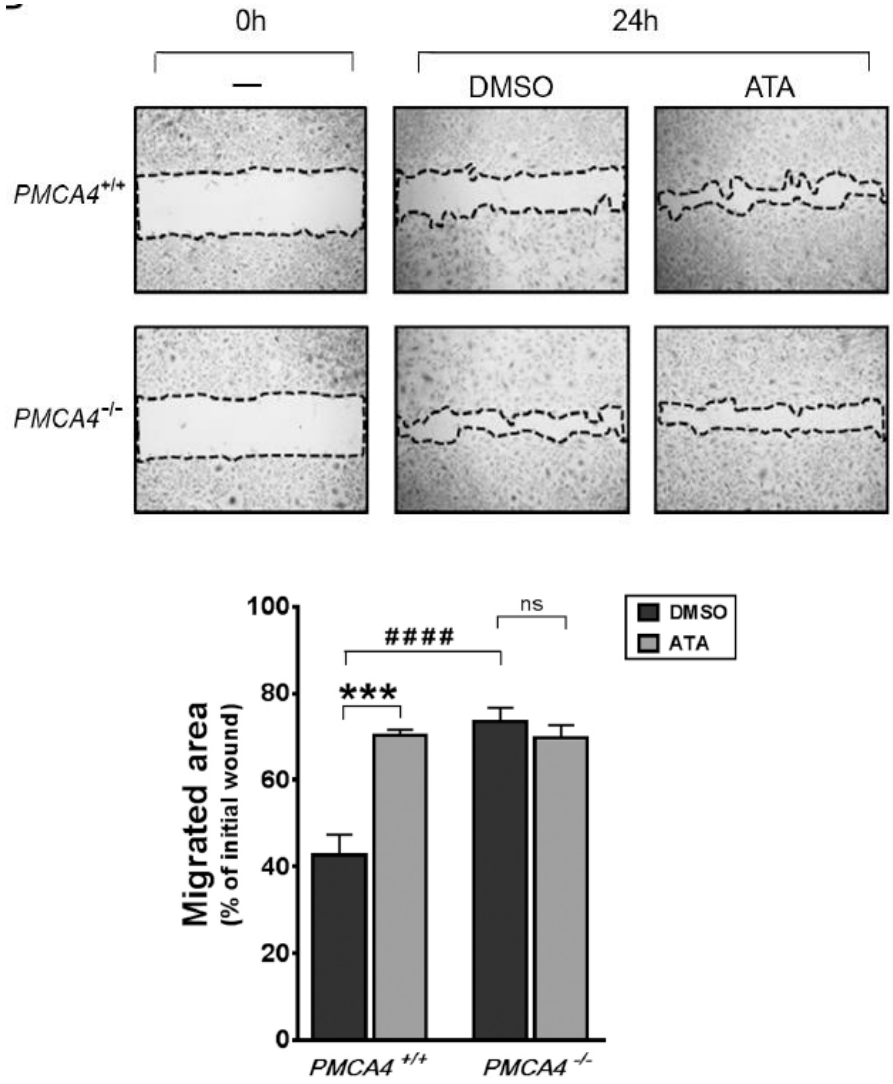


Figure 4.4.2.2 Successful isolation of *PMCA4*$^{+/+}$ or *PMCA4*$^{-/-}$ MLEC. A) MLEC cells isolated from *PMCA4*$^{+/+}$ or *PMCA4*$^{-/-}$ mice were cultured in 6 well plates for 24 hours. Protein samples were analysed by western blot using anti-PMCA4 (JA9) monoclonal antibody. Expression levels of Tubulin (α-Tub) were used as loading control. B) RNA was extracted from *PMCA4*$^{+/+}$ or *PMCA4*$^{-/-}$ MLEC cells and reverse transcribed into cDNA. *PMCA4* mRNA expression was determined by qRT-PCR using TaqMan Gene Expression Assays. Absence of *PMCA4* RNA amplification (left panel) in qPCR reactions carried out with samples reverse-transcribed with RNA isolated from *PMCA4*$^{-/-}$ MLEC confirmed loss of PMCA4 expression in these cells. *GAPDH* amplification (right panel) confirmed equal amount of cDNA in samples originated from wild-type or *PMCA4* knockout MLEC cells.

Isolated *PMCA4*$^{+/+}$ and *PMCA4*$^{-/-}$ MLEC cells were plated as described in section 4.4.1 and migration was analysed after 24 hours of stimulation with VEGF (50 ng/ml). Consistent with our published observations (Baggott *et al.,* 2014) PMCA4-deficient cells migrated longer than their corresponding wild-type counterparts (Figure 4.4.2.3). However, whereas ATA improved (64.9% increment) the ability of wild-type MLECs to

migrate, it did not have any effect at all on the migration of PMCA4-defficient cells (Figure 4.4.2.3). These results imply PMCA4 in the enhancement of endothelial cell motility triggered by ATA.



4.4.2.3 PMCA4 inhibition by ATA enhances MLEC motility in *PMCA4*$^{+/+}$ (wildtype) but not in *PMCA4*$^{-/-}$ (knockout) cells. Migration of MLEC cells was analysed at time zero or after incubation in MLEC medium supplemented with VEGF (50 ng/ml) in the presence of ATA (250 nM) or DMSO for 24 hours. Images are representative of wound healing migration assays at 0 and 24 hours. Cell migration was determined by subtracting the values of the non-migrated area from the initial wound area (time 0), and expressing this value as a percentage of the total area at time zero. Histogram shows data as mean ± SE, n=6, obtain from 3 independent experiments. Differences were analysed for statistical significance by one-way ANOVA with *post hoc* Tukey´s comparison test. ***indicates statistically significant differences ($P \leq 0.005$) in the migration of *PMCA4*$^{+/+}$ MLECs treated with ATA *vs* vehicle (DMSO). $^{\#\#\#\#}$denotes statistically significant differences ($P \leq 0.001$) in the motility of wild-type (+/+) *vs* PMCA4 knockout (-/-) MLECs. ns=non-significant.

In this study, we show that treatment with ATA impairs the molecular association between PMCA4 and calcineurin in endothelial cells (Figure 4.3.1 and 4.3.2). To investigate whether the ATA-mediated enhancement in endothelial cell motility involves disruption of the PMCA4/calcineurin interaction, we carried out cell migration assays using HUVECs where the PMCA4/calcineurin interaction was already disrupted by overexpression of the interaction domain of PMCA4 that interacts with calcineurin. We have previously reported that ectopic expression of the region 428-651 of PMCA4b, that corresponds to the domain of PMCA4 implicated in the interaction with calcineurin (Buch *et al.,* 2005), disrupts the PMCA4/calcineurin interaction in endothelial cells (Baggott *et al.,* 2014). Therefore, we infected HUVEC cells with Ad-ID4, an adenovirus encoding a Flag-tagged version of the fragment encompassing aminoacids 428 to 651 of human PMCA4 (from now on this domain of PMCA4 will be reffered to as ID4), and carried out migration assays in the infected cells. As a control, HUVECs were infected with an adenovirus encoding β-Galactosidase (Ad-LacZ). In agreement with our results presented in Figure 4.4.1 ATA boosted (64.9% increment) the migration of control cells infected with Ad-LacZ (Figure 4.4.2.4). Disruption of the PMCA4/calcineurin interaction in HUVECs infected with Ad-ID4 led to an increase in the migration of the cells as we have previously reported (Baggott *et al.*, 2014), but, conversely to our result in control cells, treatment with ATA did not further improve the migration of these cells (Figure 4.4.2.4). Equal expression of the PMCA4-interaction domain (ID4) in cells treated with DMSO or ATA confirmed that ATA treatment did not alter the infection efficiency of the Ad-ID4 adenovirus (Figure 4.4.2.5). These data, not only reassure the implication of PMCA4 on the enhancement of endothelial cell migration triggered by ATA, but also reveal that disruption of the interaction between PMCA4 and calcineurin is a key molecular mediator of this process.

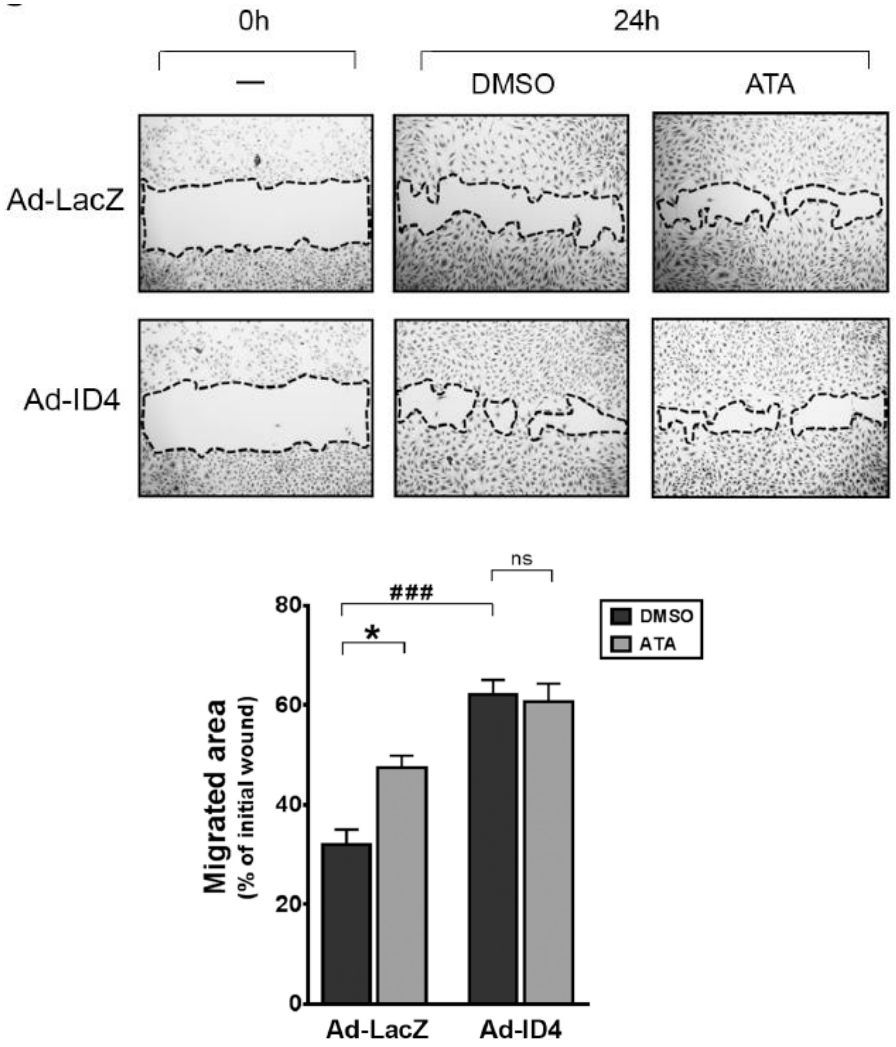


Figure 4.4.2.4 ATA increases endothelial cell motility *via* disruption of the PMCA4/calcineurin interaction. HUVEC cells were infected with Ad-LacZ or Ad-ID4 and plated in pre-coated 24-well tissue culture plates using a 0.9 mm gap generator from a wound healing assay kit to create a 0.9mm gap. Images, taken at time zero and after incubation for 24 hours are representative of 3 independent experiments. Cell migration was determined by subtracting the value of the non-migrated area from the initial wound area (time=0), and expressing this value as a percentage of the total area at time zero. Data are plotted as mean ± SE, n=3, obtained from 3 independent experiments. Differences were analysed for statistical significance by one-way ANOVA with *post hoc* Tukey´s comparison test. *indicates statistically significant differences ($P \leq 0.05$) in the motility of Ad-LacZ-infected cells treated with ATA *vs* vehicle (DMSO). $^{###}$denotes statistically significant differences ($P \leq 0.005$) in the migration of cells infected with Ad-LacZ *vs* Ad-ID4. ns=non-significant.

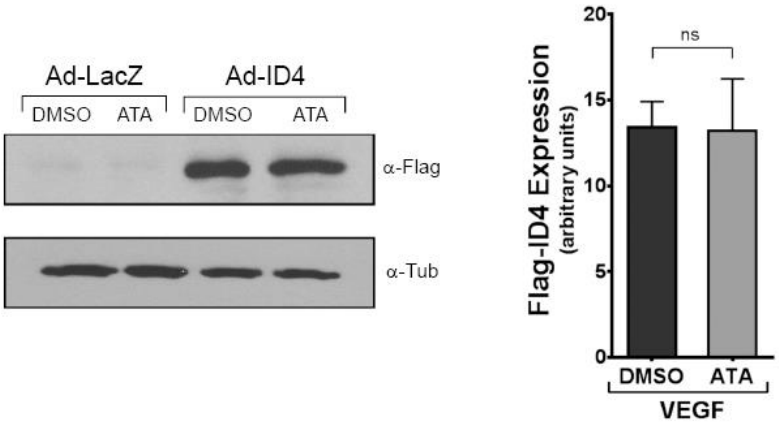


Figure 4.4.2.5 ATA does not alter the adenovirus-mediated expression of Flag-ID4. HUVECs infected (MOI=150) with Ad-LacZ (encoding β-Galactosidase) or Ad-ID4 (encoding a Flag-tagged version of the domain of PMCA4 implicated in the interaction with calcineurin) were incubated in medium containing VEGF (25 ng/ml) in the presence of ATA (250 nM) or vehicle (DMSO). Samples were analysed by western blot using anti-Flag M2 monoclonal antibody. Expression levels of Tubulin (α-Tub) were determined as loading control. Histogram shows data as mean ± SE, n=3, obtained from 3 independent experiments. Results were analysed for statistically significant differences by unpaired, two-tailed Student *t* test. ns=non-significant.

4.5 (Aim 5) To determine the effect of ATA-mediated inhibition of PMCA4 on endothelial cell tubular morphogenesis.

It is well established that both, PMCA4 and the calcineurin/NFAT pathway actively participate in the regulation of blood vessel formation (Baggott *et al*., 2014; Hernandez *et al.,* 2001). Thus, we next studied the effect of ATA on endothelial cell tubular morphogenesis. In agreement with previous reports, treatment with VEGF increased the ability of endothelial cells to form tubular-like structures in Matrigel assays (Figure 4.5A). ATA on its own did not have any effect on endothelial cell tubular morphogenesis (Figure 4.5A). However, VEGF-ATA cotreatment further enhanced the VEGF-mediated increase in tube formation (38.9% increment with respect to control cells) (Figure 4.5.1A). ATA did not increase tubular morphogenesis in cells stimulated with basic Fibroblast Growth Factor (bFGF) (Figure 4.5B), a pro-angiogenic factor that does not activate the calcineurin/NFAT pathway (Hernandez *et al*., 2001), denoting a critical role

of the calcineurin/NFAT pathway in the enhancement of tubular morphogenesis induced by ATA.

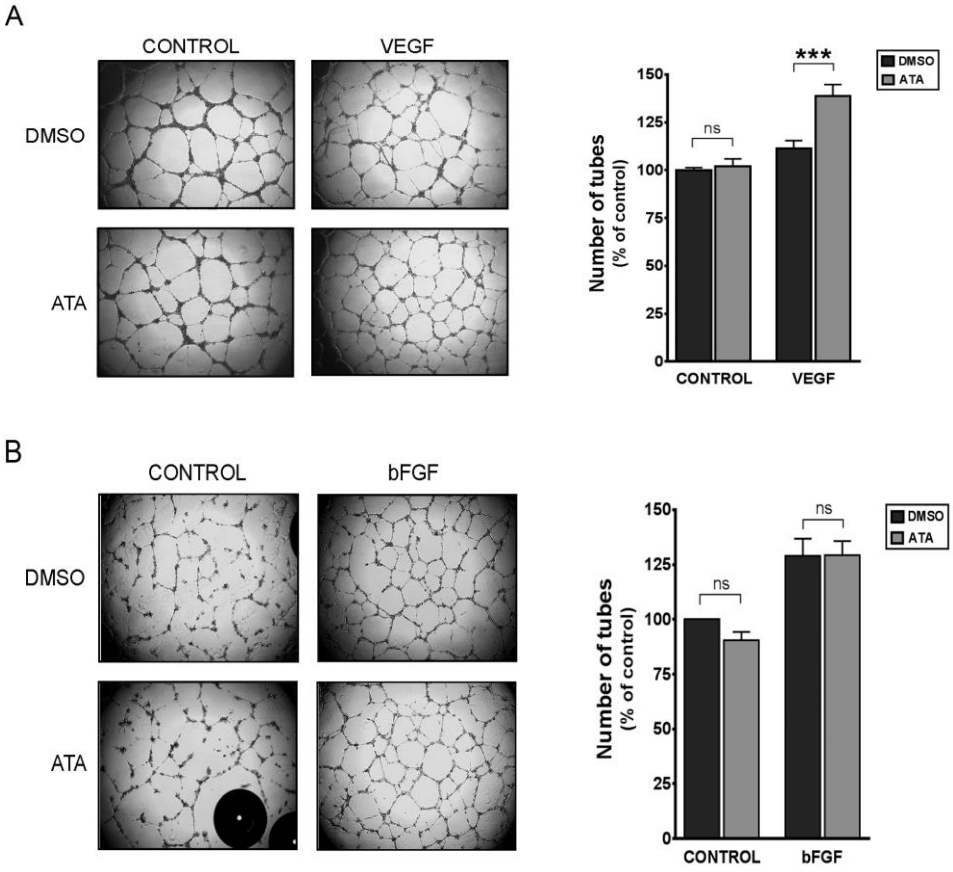


Figure 4.5 ATA enhances endothelial cell tubular morphogenesis in response to VEGF but not FGF stimulation. HUVECs plated on a layer of Growth-Factor Reduced Matrix (Geltrex) in Medium 200 containing 2% fetal bovine serum were treated when indicated with VEGF (25 ng/ml) (A) or bFGF (50 ng/ml) (B) in the presence of ATA (250 nM) or vehicle (DMSO). Representative images are shown. Histograms show data as mean ± SE, n=6 in (A) and n=4 in (B), obtained from 3 (A) and 2 independent experiments (B) respectively. Data were analysed for statistically significant differences by one-way ANOVA with *post hoc* Tukey's comparison test. ***indicates statistically significant differences ($P \leq 0.005$) when comparing tube formation in VEGF-stimulated cells treated with ATA *vs* vehicle (DMSO). ns=non-significant.

To substantiate the significance of the enhancement in blood vessel formation induced by ATA-mediated inhibition of PMCA4, our collaborators Dr Dolores Lopez-Maderuelo and Professor Juan Miguel Redondo (Centro de Investigaciones Cardiovasculares, CNIC, Madrid, Spain) have evaluated the effect of ATA *in vivo* in the mouse hindlimb ischaemia model. ATA (or DMSO as control) was administered intraperitoneally to mice undergoing experimentally-induced unilateral ischaemia by femoral artery ligation in the

lower limb. Laser Doppler analysis of blood flow revealed a robust increment in the reperfusion of post-ischaemic limbs treated with ATA. These results, shown in appendix 3 (Figure Appendix 3A) with the permission of Dr Dolores Lopez-Maderuelo and Professor Juan Miguel Redondo, not only confirm our observation but also highlight the potential of targeting endothelial PMCA4 with therapeutic purposes to treat patients suffering from cardiovascular ischaemic disease.

4.6 (Aim 6) To examine the effect of ATA on the viability of endothelial cells.

Our observations suggest that ATA might be used with therapeutic purposes to promote blood vessel formation in pathological settings occurring with insufficient angiogenesis. As a first step to evaluate this possibility we examined whether ATA has any toxic effect on the viability of endothelial cells. For this purpose, we cultured HUVECs for 3 and 6 days in the presence of 250 nM ATA or vehicle (DMSO) and determined the viability of the cells performing MTT assays. At low concentrations (250 nM) ATA did not alter the proliferation/viability of the cells (Figure 4.6.1A).

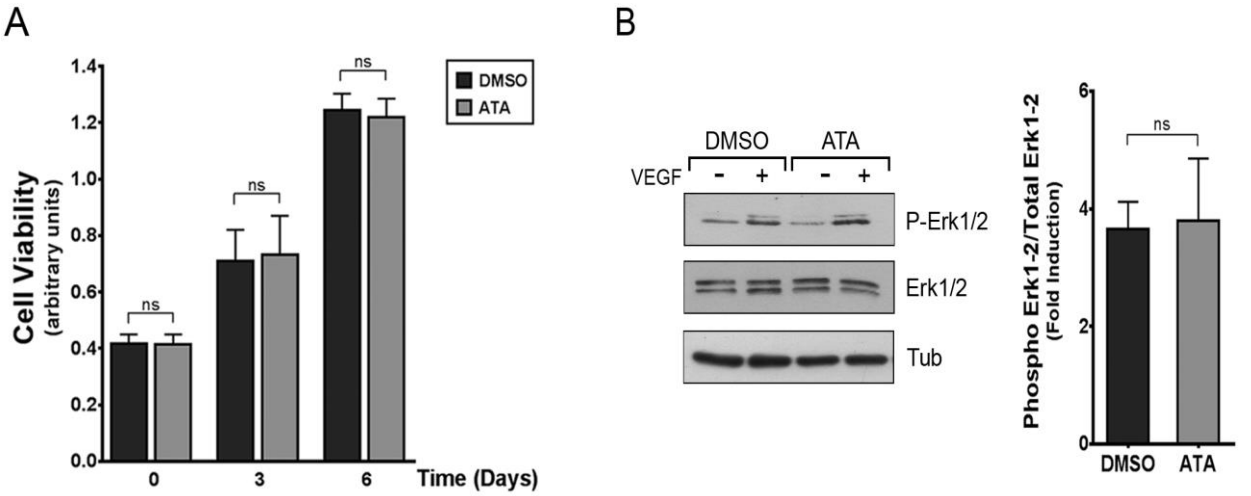


Figure 4.6.1 ATA is not toxic to endothelial cells at low concentration. HUVECs were plated on 96-well plates and cell viability determined by MTT assay after overnight incubation (t=0) and after incubation for 3 and 6 days in culture medium supplemented with VEGF (25 ng/ml) in the presence of ATA (250 nM) or vehicle (DMSO). Histogram shows data as mean ± SE, n=3, obtained from 3 independent experiments. (B) Western blot analysis of the phosphorylation (activation) status of Erk1-2 proteins using an anti-Phospho-specific-Erk1-2 antibody in HUVECs stimulated when indicated with VEGF for 5 minutes in the presence of ATA

(250 nM) or vehicle (DMSO). Histogram represents data as mean ±SE, n=5, obtained from 3 independent experiments. Phosphorylation levels of Erk1-2 were normalized to the amount of total Erk1-2 present in each sample. Data are expressed as fold-induction in Erk1-2 phosphorylation respect to that found in unstimulated cells. ns=non-significant.

In concurrence with these results, treatment with 250 nM ATA did not affect the VEGF-induced phosphorylation (activation) status of Erk1/2 proteins in endothelial cells (Figure 4.6.1B), indicating that low concentrations of ATA do not modify the activity of this proliferative signal transduction pathway.

Increasing the concentration of ATA to 20 µM had, however, a negative effect on cell viability when HUVECs were exposed to the drug for 2 weeks (Figure 4.6.2A).

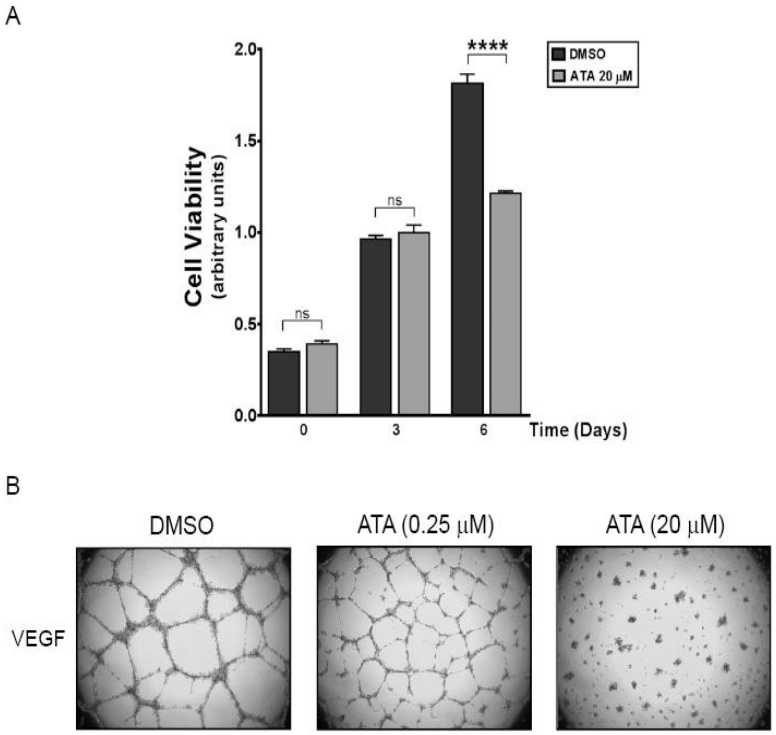


Figure 4.6.2 HUVECs exposure to high ATA levels impairs cell viability and tube formation. (A) HUVECs were incubated in endothelial cell medium containing 20 µM ATA or vehicle (DMSO) for 1 week, changing medium every 2-3 days. Subsequently, cells were seeded on 96-well plates and viability determined by MTT the day after plating cells (t=0) and after incubation for 3 and 6 days in medium supplemented with VEGF (25 ng/ml), and 20 µM ATA or vehicle (DMSO). Histogram shows data as mean ± SE, n=4, obtained from 4 independent experiments. Data were analysed for statistically significant differences by one-way ANOVA with *post hoc* Tukey's comparison test. ****indicates statistically significant differences ($P \leq 0.001$) when comparing cells incubated in the presence of 20 µM ATA *vs* vehicle (DMSO). ns=non-significant. (B) HUVECs were incubated in medium containing 0.25 µM ATA, 20 µM ATA or

vehicle (DMSO) for 1 week, changing medium every 2-3 days. Subsequently, cells were seeded on a layer of Growth-Factor Reduced Matrix (Geltrex) in Medium 200 containing 2% fetal calf serum, VEGF (25 ng/ml) and 0.25 μM ATA, 20 μM ATA or vehicle (DMSO) and incubated for 24 hours longer. Images are representative of two independent experiments.

Likewise, high levels of ATA (20 μM) completely abolished the ability of VEGF-stimulated HUVECs to form capillary-like structures in matrigel assays (Figure 4.6.2B). In agreement with the harmful effects exerted by high concentration of ATA observed in our in vitro assays using endothelial cells (Figure 4.6.2), our collaborators Mr Aaron Savage and Dr Robert Wilkinson (Department of Infection, Immunity and Cardiovascular Disease and Bateson Centre, University of Sheffield, UK) have found that treating zebrafish embryos with ATA at low concentration caused no significant damage to the embryos which develop a normal vascular anatomy. However, increasing the concentration of ATA above 100 μM caused embryo death. These results, shown in appendix 3 (Figure Appendix 3B) with the permission of Mr Aaron Savage and Dr Robert Wilkinson confirm in a more physiological setting the *in vitro* data obtained in primary endothelial cells.

4.7 (Aim 7) To determine the effect of ATA on the interaction of PMCA4 with endothelial nitric oxide synthase (eNOS).

Here, we demonstrate that the calcineurin/NFAT signalling pathway has a key role in the enhancement of VEGF-induced angiogenesis promoted by inhibition of PMCA4 with low concentrations of ATA, however, our results do not rule out the potential participation of other signalling pathways. In addition to calcineurin, we have recently shown that PMCA4 also downregulates the activity of eNOS, and the subsequent production of nitric oxide (NO) in endothelial cells (Holton *et al*., 2010). The pro-angiogenic properties of NO are well established, and eNOS activation and NO production is induced by endothelial cell stimulation with VEGF. Thus, it is tempting to

speculate that NO-dependent signalling pathways might also be implicated in the angiogenic enhancement exerted by ATA in VEGF-stimulated endothelial cells. To investigate this possibility, we first determined whether exposure of endothelial cells to ATA leads to disruption of the PMCA4/eNOS interaction in a similar manner to the effect observed for the PMCA4/calcineurin interaction. However, when we immunoprecipitated PMCA proteins from lysates of HUVECs treated with VEGF and ATA (or DMSO as control) and analysed the levels of co-precipitated eNOS, we observed that ATA led to an increment in the level of eNOS associated to PMCA4 (Figure 4.7A). As we have previously reported that the interaction between PMCA4 and eNOS results in downregulation of eNOS activity (Holton *et al*., 2010), we next studied the functional effect of ATA on the synthesis of nitric oxide (NO) by endothelial cells stimulated with VEGF. As we could anticipate, ATA treatment significantly diminished the synthesis of NO in HUVECs stimulated with VEGF (Figure 4.7B). The reduction in NO synthesis promoted by ATA is likely to be the result of enhancement of the PMCA4/eNOS interaction.

Altogether, these results seem to exclude NO as one of the effectors implicated in the boost of VEGF-induced angiogenesis promoted by inhibition of PMCA4 with ATA.

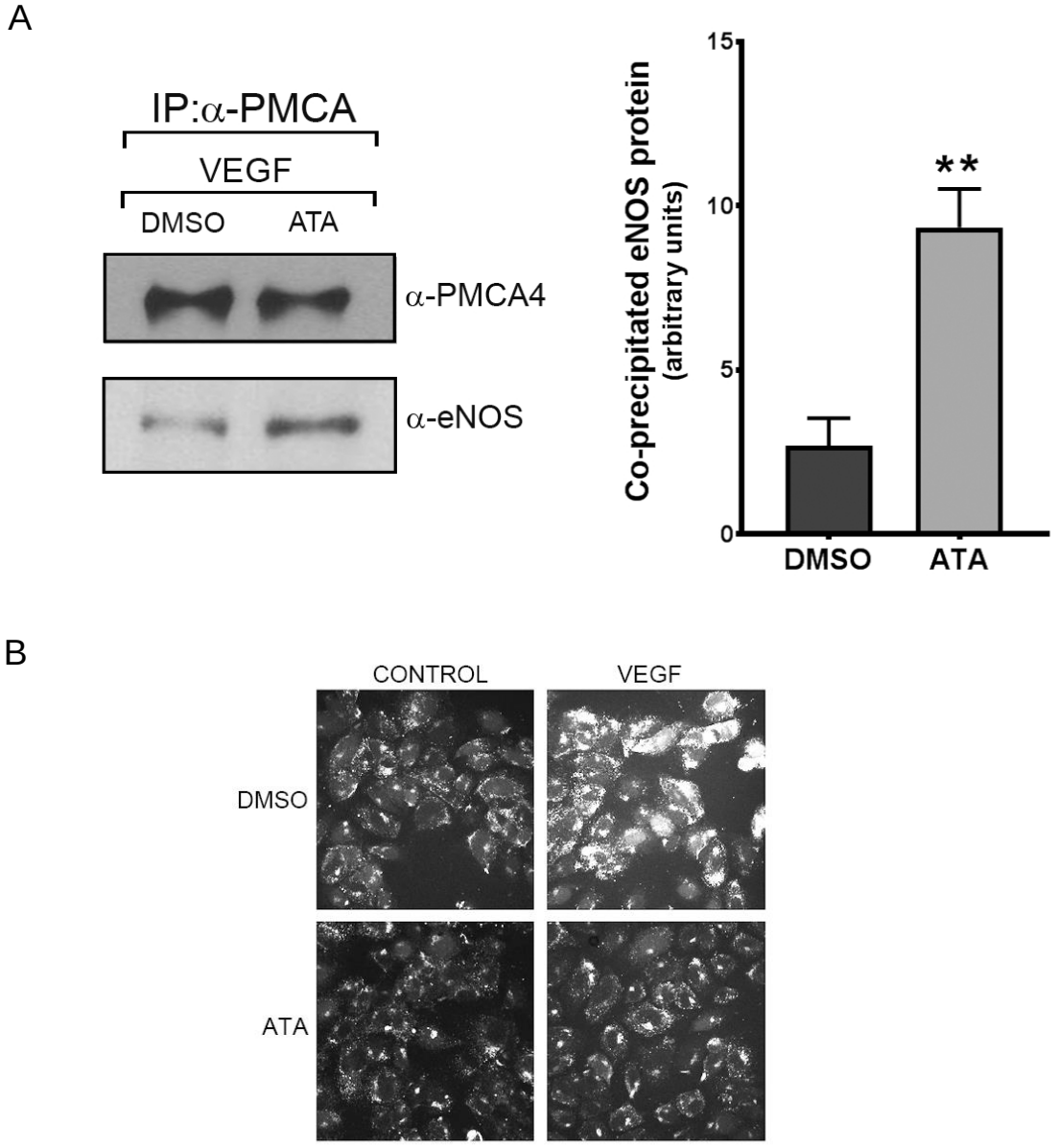


Figure 4.7 ATA treatment potentiates the PMCA4/eNOS interaction and attenuates nitric oxide synthesis in endothelial cells stimulated with VEGF. (A) Serum-starved HUVECs were treated with VEGF (25 ng/ml) and ATA (250 nM) or vehicle (DMSO) for 5 minutes. Protein lysates were immunoprecipitated with the anti-PMCA 5F10 monoclonal antibody, and levels of PMCA4 and eNOS in the immunoprecipitated proteins analysed by western blot. Histogram shows levels of co-precipitated eNOS normalised to the amount of PMCA4 precipitated in each assay. Data are shown as mean ± SE obtained from 3 independent experiments. **indicates statistically significant differences ($P \leq 0.01$, according to unpaired, two-tailed Student *t* test) in the levels of eNOS co-precipitated in samples treated with ATA *vs* vehicle (DMSO). Western blot images are representative of 3 independent immunoprecipitation experiments. (B) HUVECs were treated as indicated in (A). Intracellular levels of nitric oxide were determined by staining cells with the NO sensitive dye DAF-FM. Images showing microscopy fields of DAF-FM staining in cells are representative of 2 independent experiments performed in duplicate.

CHAPTER 5

Discussion and Future work

5.1 Discussion

Ischaemic cardiovascular diseases such as ischaemic heart disease, peripheral arterial disease, and stroke, constitute a leading primary cause of morbidity and mortality worldwide (Mozaffarian *et al*., 2015). During the last decades, the treatment for ischaemic cardiovascular disease has advanced into an inspiring new era. Bypass surgery and interventional endovascular procedures are currently applied to patients with ischaemic cardiovascular disease to successfully restore the flow of blood in the ischaemic tissues (Al Sabti, 2007; Sachs *et al.,* 2011). However, in many cases, the extensive distribution and diffuseness of arterial occlusions makes a high proportion of patients unsuitable for surgical revascularisation (Sachs *et al.,* 2011). Therapeutic interventions aimed to induce the formation of new blood vessels in the ischaemic organs (referred to as therapeutic angiogenesis) represent a very promising alternative to treat these patients.

Given the relevance of the pro-angiogenic factor VEGF as a pivotal mediator of blood vessel formation (that we have described in detail in the introduction section of this thesis), it is not surprising that a number of gene-based and protein-based therapeutic approaches have been developed in the last 10 years to deliver exogenous VEGF to ischaemic tissues (Zachary and Morgan, 2011). An impressive body of data obtained from preclinical studies carried out in animal models of myocardial and limb ischaemic disease showed that ectopic delivery of VEGF led to successful reperfusion of the ischaemic organ (Ylä-Herttuala, 2013; Zachary and Morgan, 2011). Unfortunately, translation of these procedures onto treatment of human cardiovascular ischaemic diseases have only shown limited benefits for patients thus far (Gupta *et al.,* 2009). The reasons behind lack of success in trials of treatments based on therapeutic angiogenesis are complex, but one major problem in current interventions is failure to reinstate efficient VEGF activity in the ischaemic human tissue (Ylä-Herttuala, 2013;

Zachary and Morgan, 2011; Gupta *et al.,* 2009). Therefore, there is an urgent need to develop new therapeutic approaches that improve VEGF-based blood vessel formation in patients with occluded arteries. A full understanding of the molecular mechanisms that regulate reparative angiogenesis and collateral development in ischaemic tissues is an essential requirement for the design of novel, more efficient therapeutic interventions that promote reparative revascularization. The work presented in this thesis has established proof-of-concept for the potential of targeting endothelial PMCA4 to promote blood vessel formation with therapeutic purposes. Pharmacological suppression of PMCA4 function seems a feasible strategy for therapeutic interventions in clinic, as PMCA4 deficiency in mouse knockout models does not cause any pathological phenotype in the animal apart from male infertility (Such *et al*., 2005).

In this work we demonstrate that inhibition of PMCA4 function with low concentrations of the small polyaromatic compound aurintricarboxylic acid (ATA) increases VEGF-mediated angiogenesis *in vitro*. Consistently with our *in vitro* data obtained using cultured primary human endothelial cells, our collaborators Dr Dolores Lopez-Maderuelo and Professor Juan Miguel Redondo (Centro de Investigaciones Cardiovasculares, CNIC, Madrid, Spain) have evaluated in pre-clinical animal models the potential of using ATA to promote blood vessel formation in ischaemic organs. Their results, presented in appendix 3A (with permission of Dr Lopez-Maderuelo and Professor Juan Miguel Redondo) show that administration of ATA significantly enhances the reperfusion of ischaemic limbs in a murine animal model of experimental hind limb ischaemia (Figure Appendix 3A, appendix 3) indicating the potential application of ATA in pro-angiogenic therapies. The feasibility of ATA in clinic has yet to be tested but pre-clinical studies using animal models have revealed potential clinical applications for ATA in the treatment of cardiac hypertrophy (Mohamed *et al.,* 2016), experimental autoimmune encephalomyelitis (Zhang *et al.,* 2013), sepsis (Laufenberg *et*

al., 2014), or myocardial ischaemia-reperfusion injury (Zhao *et al.,* 2003). However, the results from this thesis anticipate some problems (dosage and duration of treatment) for the clinical use of ATA in its present formulation. Our data demonstrate that potential therapeutic strategies based on ATA administration should take into account the harmful effects of the drug observed at high doses and in prolonged treatments. We show that high concentration of ATA (20 µM) has a negative effect on endothelial cell viability and completely abolishes VEGF-stimulated tube formation when HUVECs are exposed to the drug for two weeks. Consistent with this finding, previous studies by other groups have shown that increasing the concentration of ATA diminishes the ability of endothelial cells to form tubular-like structures in matrigel assays (Lipo *et al.,* 2013). In the same work, the authors also demonstrated that injection of 1.5µg of ATA in 2µl of 10% DMSO (that corresponds to a concentration 1.78 mM) by intravitreal injection into a mice model of age-related macular degeration (AMD), strongly attenuates laser-induced choroidal neovascularization. It is likely that the reduction in ocular vascularization observed in the work by Lipo et al, is due to the toxic effect exerted by high concentrations of ATA on endothelial cells. Supporting this possibility, experiments in zebrafish embryos performed by our collaborators Mr Aaron Savage and Dr Robert Wilkinson (University of Sheffield) (Figure Appendix 3B, appendix 3) and those performed by Smee *et al* in mice (Smee *et al.,* 2010), clearly demonstrate the harmful effects exerted by high concentrations of ATA. Furthermore, other studies have shown that high concentrations of ATA (5-20 µM) act as an inhibiting agent for the growth of fibroblasts (Benezra *et al*., 1992) and smooth muscle cells (Benezra *et al.,* 1994). At high concentrations, ATA has been reported to inhibit non-specifically the action of nucleases (Hallick *et al.,* 1997), the enzyme calpain (Posner *et al.,* 1995), several chemokine receptors (Zhang *et al.,* 2013), and other molecules, offering a plausible explanation for the toxic effect associated to high concentrations of ATA. For this

reason, in this study we used a very low dose of ATA (250 nM) to ensure that its effects are only due to PMCA4 inhibition, and to avoid the toxicity associated to higher concentrations.

Although the associated toxicity of ATA can preclude its clinical value at present, we have found in this work that ATA-mediated inhibition of PMCA4 can successfully enhance angiogenic processes, and, therefore, refined, more specific versions of ATA free of toxic effects might be used in the future to improve pro-angiogenic treatments for patients. This possibility requires further investigation.

Our results suggest that ATA increases calcineurin-dependent angiogenic processes through the disruption of the interaction between PMCA4 and calcineurin at the plasma membrane. The molecular mechanism by which ATA promotes disruption of the PMCA4/calcineurin complex is not known yet. A possible explanation is that when ATA binds to PMCA4 to inhibit its calcium extrusion activity, it also masks the area of PMCA4 involved in the interaction with calcineurin (amino acids 428 to 651 of human PMCA4b) (Buch *et al.,* 2005). Supporting this possibility, Shadrick *et al* demonstrated that ATA inhibits the action of the hepatitis C virus helicase by masking the interaction domain between the enzyme and nucleic acids (Shadrick *et al.,* 2013). However, we have also investigated the effect of ATA on PMCA4 binding to the endothelial nitric oxide synthase (eNOS), another partner protein that interacts with the same domain of PMCA4 as calcineurin (Holton *et al*, 2010; Buch *et al*., 2005), and we have found that rather than decreasing the interaction, ATA enhances the association between PMCA4 and eNOS (Figure 4.7.1). As ATA has opposite effects on the binding of PMCA4 with calcineurin or eNOS, it is unlikely to be an allosteric competitor with calcineurin or eNOS for binding to the domain 428 to 651 of PMCA4. Further research is necessary to fully define the molecular events through which ATA disrupts the PMCA4/calcineurin interaction, and the differential effect exerted by ATA on PMCA4 binding to calcineurin or eNOS.

The results presented on this thesis highlight the relevance of PMCA4 over other PMCA isoforms on the regulation of endothelial cell angiogenesis. As reviewed by Strehler and Zacharias (2001), there are four isoforms of PMCA (PMCA1-4) encoded by four different genes. PMCA1 and 4 are found throughout most tissues (Strehler and Zacharias, 2001). This work and a previous report by Holton *et al*., 2010 has shown the expression of PMCA1 and 4 in HUVEC and Human Dermal Microvascular Endothelial Cells (HDMEC). The expression of these proteins in other vascular beds like for example in aortic endothelial cells, pulmonary artery endothelial cells, coronary artery endothelial cells, etc has not been investigated so far. ATA has been shown to inhibit PMCA4 with no significant effect on PMCA1 (Mohamed *et al*., 2013). Therefore, although PMCA4 will be inhibited in cells treated with ATA, PMCA1 will still be fully functional in these cells. Our results indicate that ATA-mediated inhibition of PMCA4 is enough to enhance calcineurin-dependent blood vessel formation even in the presence of functional PMCA1. Although it had been traditionally considered that all PMCA isoforms exert a similar function as membrane extruders of cytoplasmic calcium, emerging evidence suggests that specific isoforms fulfil more specialised roles (Strehler, 2015; Little *et al*., 2016), and our observations are in line with this idea.

Here, we show that treatment of endothelial cells with nanomolar concentrations of ATA notably enhances calcineurin/NFAT signalling pathway, and subsequently promotes VEGF-mediated angiogenesis. However, this finding does not rule out the activation by ATA of other signalling pathways involved in VEGF-mediated angiogenesis. In addition to calcineurin, we have recently reported that PMCA4 also downregulates the activity of eNOS and the subsequent production of nitric oxide (NO) in endothelial cells (Holton *et al* 2010). NO has been identified as a positive inducer of angiogenesis (Luque Contreras *et al.,* 2006). Furthermore, activation and NO production are stimulated by treatment of endothelial cells with VEGF (Ziche and Morbidelli, 2000). Therefore, NO-

dependent signalling pathways might also be implicated in the angiogenic enhancement exerted by ATA in VEGF-stimulated endothelial cells. However, we noted in this study that targeting PMCA4 with ATA increases the interaction between PMCA4 and eNOS in the presence of VEGF (Figure 4.7.1A), and, given the negative effect exerted by PMCA4 on eNOS (Holton *et al* 2010), this should lead to downregulation of eNOS activity. In agreement with this idea our functional experiments show that nanomolar concentrations of ATA significantly attenuate NO synthesis in endothelial cells stimulated with VEGF (Figure 4.7.1B), discarding NO as one of the effectors implicated in the boost of angiogenesis exerted by ATA. Still, we cannot rule out that interaction of PMCA4 with other signalling partner protein yet to be identified, is implicated in the regulation of VEGF-driven angiogenesis, and, thus, participates in the angiogenic enhancement induced by ATA.

5.2 Future work

In this study we have identified that inhibition of PMCA4 in endothelial cells by treatment with the PMCA-specific inhibitor ATA significantly increases the activity of calcineurin/NFAT pathway, endothelial motility, and blood vessel formation in response to VEGF stimulation. We have seen that disruption of the PMCA4/calcineurin interaction is essential for the ATA-induced enhancement of angiogenic processes, as endothelial cells where the PMCA4/calcineurin interaction is already disrupted by overexpression of the domain 428-651 of PMCA4 did not respond to ATA treatment. This result suggests that molecular strategies that disrupt the PMCA4/calcineurin interaction in endothelial cells might be used with therapeutic purposes to enhance VEGF-triggered angiogenesis in patients.

As a first step in this direction, it would be very beneficial to characterise the molecular nature of the interaction between PMCA4 and calcineurin. Previous work by our laboratory has shown the interaction between PMCA4 and calcineurin in HEK293 and

endothelial cells (Buch *et al*., 2005; Holton *et al.,* 2010; Baggott *et al.,* 2014), but these studies have not revealed whether the interaction between the two proteins is direct or it involves the participation of an adaptor protein that brings them together. It would be interesting to use recombinant versions of PMCA4 and calcineurin to perform in vitro pull-down assays in the presence of the two recombinant proteins. Furthermore, these pull-down assays could test the relevance of other co-factors such as calmodulin, calcium, etc that can be easily modified in these assays by changing the conditions of the reaction buffer.

Another interesting point to consider is that in this and in previous works by our loboratory (Buch *et al.,* 2005; Baggott *et al.,* 2014), we have used a large polypeptide of 222 amino acids that corresponds to the region 428 to 651 of PMCA4. Although this polypeptide has proven extremely useful to establish proof-of-concept on the potential of disrupting the PMCA4/calcineurin interaction in endothelial cells to boost VEGF-driven angiogenesis, clinical delivery of such a big protein in patients might turn out to be difficult. Further studies should be conducted to narrow down the domain of PMCA4 implicated in the interaction with calcineurin to the minimal size. This information might lead to the design of small cell-penetrating peptides, based on this small fragment of PMCA4, that would enter the endothelial cells and disrupt the interaction with calcineurin.

In this thesis I have also demonstrated that inhibition of the calcium-extrusion function of PMCA4 has a different effect on the interaction of the pump with calcineurin or eNOS. In fact, whereas inhibition of PMCA4 with ATA enhances angiogenic processes, ATA attenuates VEGF-induced synthesis of nitric oxide. Therefore, together with the characterisation of the molecular nature of the PMCA4/calcineurin interaction, further analysis of the PMCA4/ eNOS interaction is also warranted. Particular attention should be paid to the role play by calcium in the interaction of PMCA4 with these two partner

proteins. Inhibition of PMCA4 calcium extrusion with ATA is expected to increase the concentration of intracellular calcium in the vicinity of PMCA4. Work performed by other laboratories in human sperm has shown that the interaction between PMCA4 and eNOS increases with elevation of intracellular calcium. This might explain our observation showing increased interaction between PMCA4 and eNOS in endothelial cells treated with ATA.

Given the critical relevance of nitric oxide synthesis by endothelial cells in the control of vascular tone, future therapeutic interventions based on PMCA4 inhibition might have undesired hypertensive side-effects. For this reason, the characterisation of the molecular nature of the interaction of PMCA4 with calcineurin and eNOS is essential to the future development of efficient therapeutic strategies. Moreover, although both proteins have been reported to interact with the domain 428-651 of PMCA4, this is a very big domain. Therefore, together with the identification of the minimal domain of PMCA4 that interacts with calcineurin, it will also be highly interesting to determine the minimal region of PMCA4 that interacts with eNOS. If minimal interaction domains with the two partner proteins are different, this information could be used to disrupt the interaction PMCA4/calcineurin without affecting to the interaction PMCA4/eNOS and thus without modifying the influences of PMCA4 on nitric oxide production.

All these possibilities deserve further investigation.

5.3 Limitations of the study

Given the context of the target disease in this study, HUVEC cells were used as an *in vitro* model to study angiogenesis. HUVEC cells are a widely used, robust cell model to study molecular mechanisms of endothelial cell function (Staton *et al*., 2009). If time permitted, the effect of ATA should have been further validated in different vascular beds using other arterial cells such as Human Cardiac Microvascular Endothelial cells

(HCMEC), Human Aortic Endothelial cells (HAoEC), Human Coronary Artery Endothelial cells (HCAEC), etc.

Another interesting point to consider is that the western blot data presented in figure 4.4.2.2 shows several bands. The size of these bands (around 138 kDa) is in agreement with size reported for PMCA4, furthermore, there is no detection of any of these bands in protein extracts isolated from PMCA4 knockout mice, thus confirming the specificity of the antibody for detection of PMCA4. The presence of 3 different bands is not clear at present. Even when we have used protease inhibitors for processing the samples, the different bands could represent degradation products from the full length protein. Alternatively, these bands might be the result of alternatively spliced versions of PMCA4. As previously mentioned, RNA alternative splicing at site A and C generates huge PMCA isoform diversity. To date more than 20 varieties of PMCAs have been reported (Strehler and Zacharias., 2001). Therefore, it would be very interesting to determine the exact involvement of different splice versions of PMCA4 in the regulation of calcineurin/NFAT pathway in the presence of ATA and consequently angiogenesis in endothelial cells.

This study has established proof-of-concept on the potential therapeutic value of targeting endothelial PMCA4 to promote blood vessel formation in patients suffering from cardiovascular ischemic disease characterised by insufficient angiogenesis. Since therapeutic angiogenesis will be required in ischemic disease, endothelial cell activation and effect of ATA under hypoxic condition need to be performed. Further study into the role of PMCA4 and ATA in the regulation of signalling pathways under hypoxic condition, provides a potential therapeutic value for the regulation of pathological angiogenesis.

Concluding Remarks

- This work has established proof-of-concept on the potential therapeutic value of targeting endothelial PMCA4 to promote blood vessel formation in patients suffering from cardiovascular diseases characterised by insufficient angiogenesis.
- Inhibition of PMCA4 calcium extrusion activity in endothelial cells with low doses of the small molecule aurintricarboxylic acid (ATA) enhances VEGF-induced angiogenic processes such as endothelial cell motility and tubular morphogenesis.
- High concentrations of ATA administered for prolonged periods reduce endothelial cell viability and impair tube formation, suggesting that at its present formulation ATA is unlikely to be suitable for clinical use. Refined, less-toxic versions of ATA or the identification of novel, more specific inhibitors of PMCA4 would be required in therapeutic clinical interventions.
- ATA enhances the VEGF-induced activity of the calcineurin/NFAT pathway through disruption of the interaction between PMCA4 and calcineurin at the endothelial cell membrane. Our results indicate that novel therapeutic strategies based on the disruption of the PMCA4/calcineurin interaction in endothelial cells might have an important clinical value to improve blood vessel formation in the ischaemic tissues of patients with cardiovascular ischaemic disease.

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APPENDIX

APPENDIX 1 Table of stacking and resolving gel preparation for western blot analysis

Stacking Gel	
Stacking buffer (0.5 M Tris-HCl, 0.4% SDS, pH6.6) (GENEFLOW, UK) Ultra Pure ProtoGel® 30% (w/v)	2.5 ml
Acrylamide: 0.8% (w/v) Bis-Acrylamide (37.5:1) (acrylamide/bis-acrylamide) (GENEFLOW, UK)	1.4 ml
dH2O	6.1 ml
10% ammonium persulfate (APS)	100 µl
N,N,N',N'-Tetramethylethylenediamine (TEMED)	80 µl

Resolving Gel Solutions	**Percentage of gels**			
	6%	8%	10%	12%
Ultra Pure ProtoGel® 30% (w/v) Acrylamide: 0.8% (w/v) Bis-Acrylamide (37.5:1)(GENEFLOW, UK) (ml)	2.80 ml	3.80 ml	4.70 ml	5.60 ml
10x resolving buffer (4x 1.5 M Tris-HCl, 0.4% SDS, pH8.8) (GENEFLOW, UK) (ml)	3.70 ml	3.75 ml	3.75 ml	3.75 ml
10% ammonium persulphate (µl)	100µl	100 µl	100 µl	100 µl
H2O (ml)	7.35 ml	6.4 ml	5.45 ml	4.55 ml
N,N,N',N'-Tetramethylethylenediamine (TEMED)	80 µl	80 µl	80 µl	80 µl

APPENDIX 2 Table of primary antibodies used for western blot analysis

Primary antibody specific for	Type of antibody	Host	Dilution	Supplier
DSCR1 (RCAN1.4)	Polyclonal	Rabbit	1:1000	Sigma-Aldrich, UK
Calcineurin- A (CnA)	Monoclonal	Mouse	1:1000	BD Transduction Laboratories
PMCA4 (JA3)	Monoclonal	Mouse	1:500	Santa Cruz Biotechnology
Tubulin	Monoclonal	Mouse	1:2500	Sigma-Aldrich, UK
Pan cadherin	Polyclonal	Rabbit	1:4000	Abcam
eNOS total	Polyclonal	mouse	1:10000	Sigma-Aldrich, UK
Erk 1/2	Polyclonal	Rabbit	1:1000	Cell Signalling Technology
p-Erk 1/2	Polyclonal	Rabbit	1:1000	Cell Signalling Technology
Flag epitope (M2 peroxidase HRP-conjugated)	Monoclonal		1:2000	Sigma-Aldrich, UK
Secondary antibody			**Dilution**	**Supplier**
ECL™ Anti-Rabit IgG- Horseradish peroxidase			1:5000	GE Healthcare, UK
ECL™ Anti-Mouse IgG- Horseradish peroxidase			1:5000	Sigma-Aldrich, UK

APPENDIX 3 Experiments performed by our collaborators related to the study presented in this thesis

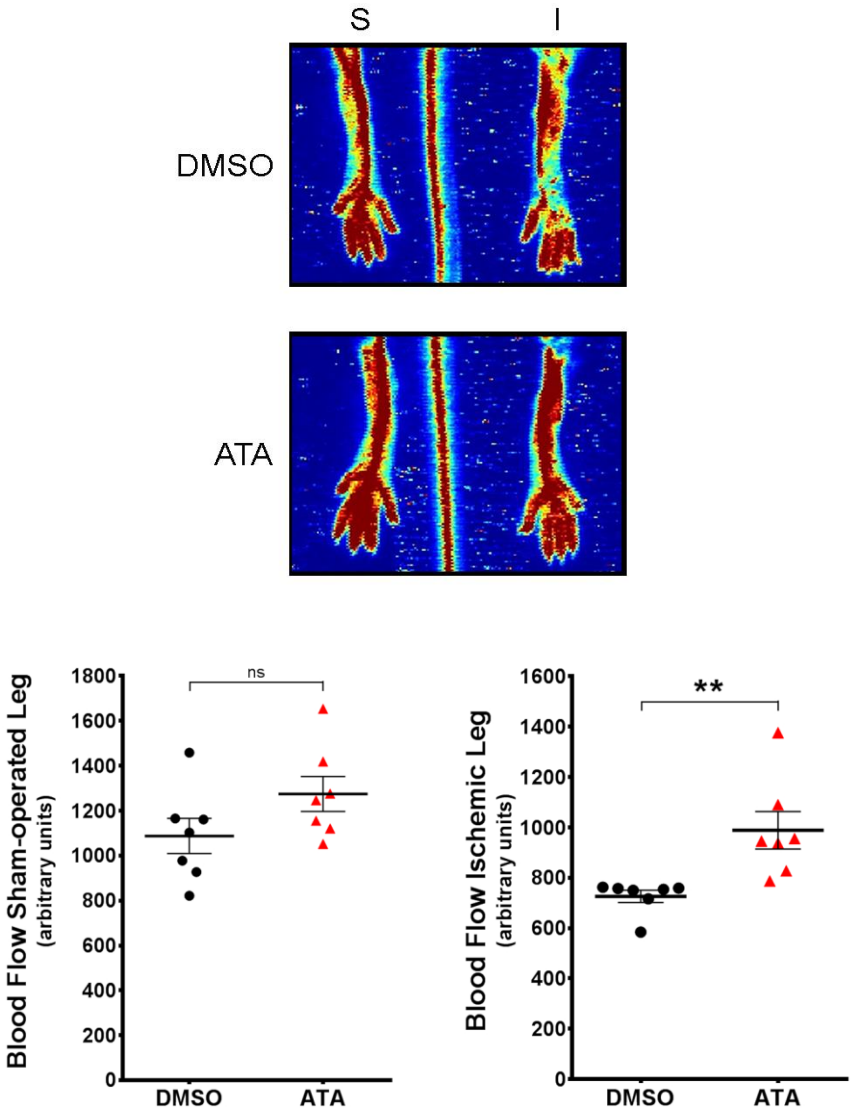


Figure Appendix 3A Administration of ATA at low concentrations boosts post-ischaemic hind limb perfusion in mice. Representative laser Doppler images showing blood flow at 4 days after surgery in sham-operated non-ischaemic legs (S) and femoral-ligated ischaemic legs (I) of mice receiving ATA (5 mg/kg/day) or vehicle (DMSO) by intraperitoneal injection. Histograms show data as mean ±SE, n=7, obtained from 2 independent experiments. **denotes statistically significant differences ($P \leq 0.01$, according to unpaired, two-tailed Student *t* test) in blood flow in the ischaemic leg of mice receiving ATA *vs* vehicle. ns=non-significant.

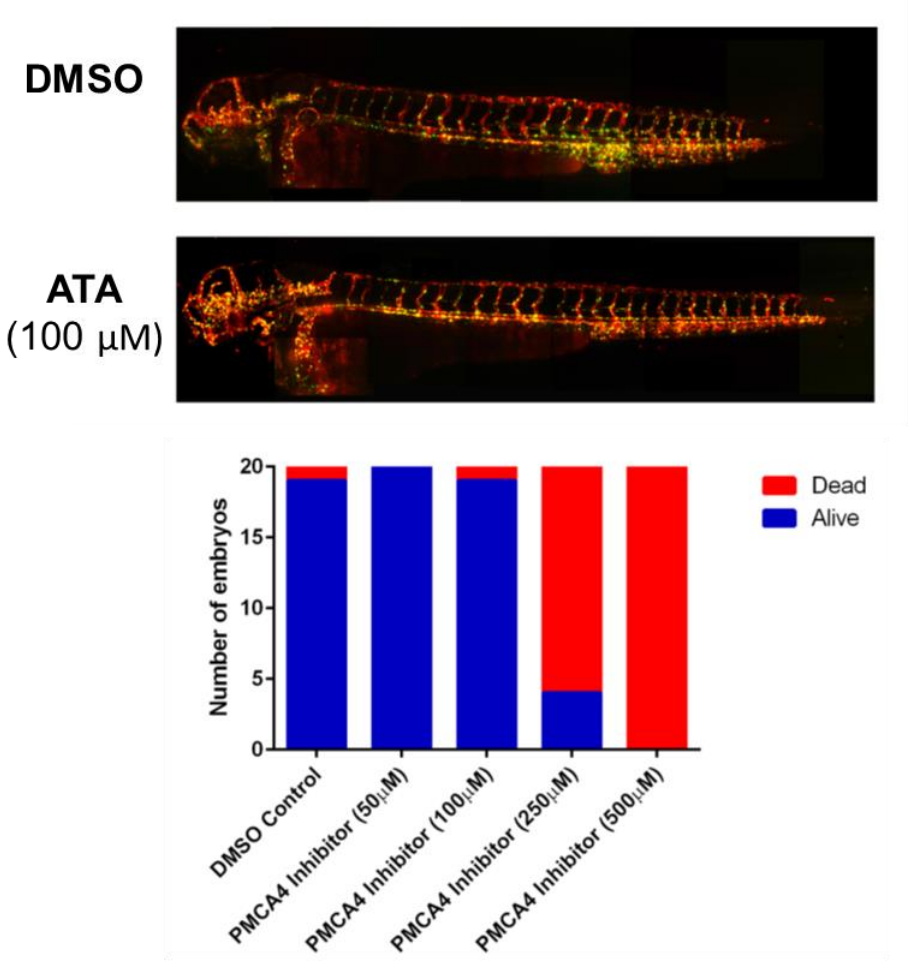
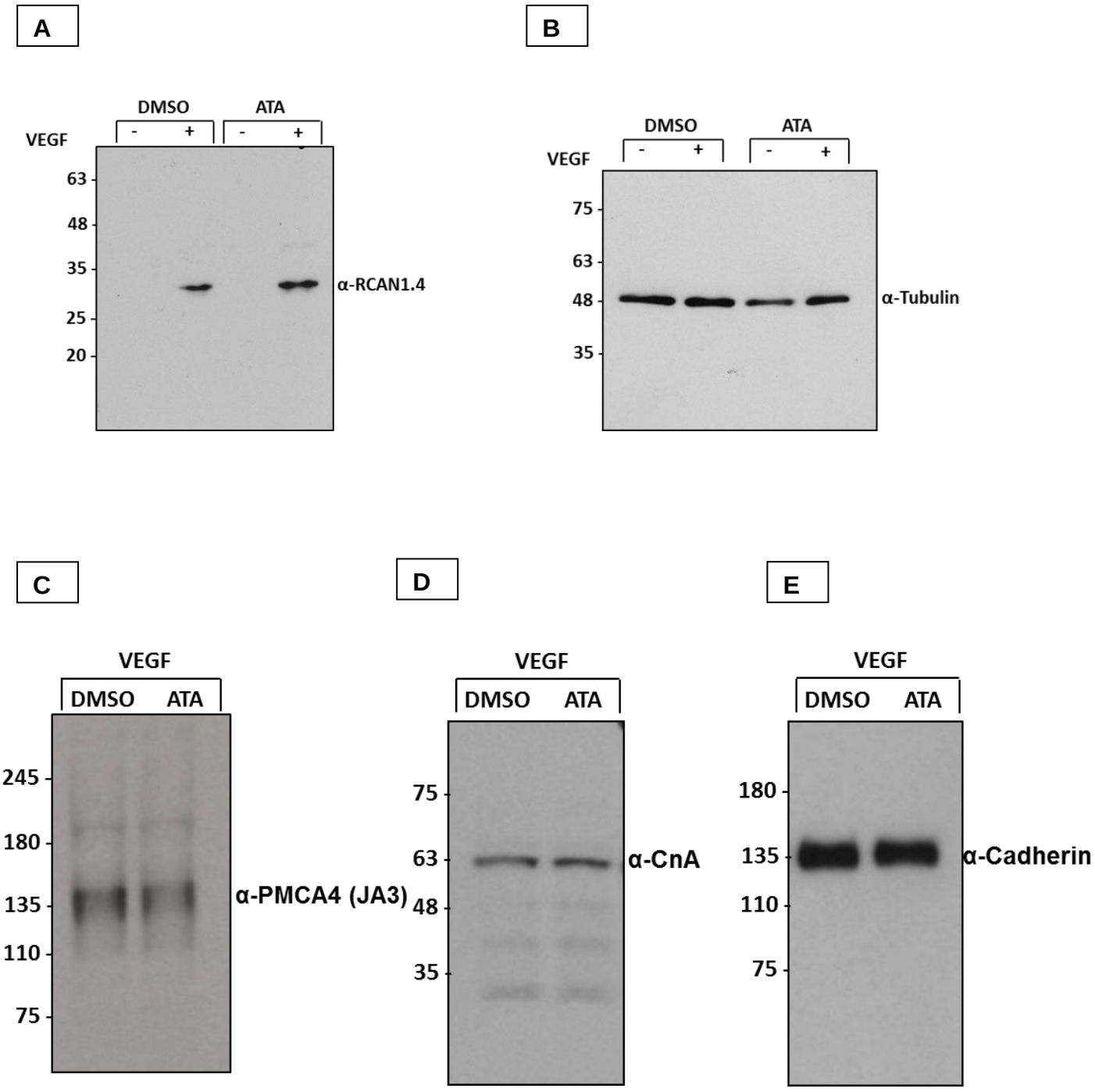


Figure Appendix 3B ATA treatment is toxic to zebrafish embryos at concentrations over 100 μM. Zebrafish Tg(kdrl:HRAS-mCherry; flk1:EGFP-nls) dechorionated embryos were incubated for 24 hours with increasing concentrations of the selective PMCA4 inhibitor ATA or vehicle (DMS0) from 30 hours post fertilisation (hpf). Vascular morphology was imaged at 54 hpf using a Zeiss Lightsheet Z.1 microscope. Images show vascular morphology in embryos treated with100 μM ATA or DMSO. Membrane-tagged endothelial mCherry (red). Endothelial cell nuclei (green). Histogram shows embryo viability 24 hours after treatment. Results are representative of 2 independent experiments.

APPENDIX 4 Determination of adenovirus plaque forming units

Dilutions	PFU/mL	Dilutions	PFU/mL
10^{-2}	1×10^{3}	7.81×10^{-9}	1.28×10^{9}
10^{-3}	1×10^{4}	3.91×10^{-9}	2.56×10^{9}
10^{-4}	1×10^{5}	1.95×10^{-9}	5.12×10^{9}
10^{-5}	1×10^{6}	9.77×10^{-10}	1.02×10^{10}
10^{-6}	1×10^{7}	4.88×10^{-10}	2.05×10^{10}
5×10^{-7}	2×10^{7}	2.44×10^{-10}	4.1×10^{10}
2.5×10^{-7}	4×10^{7}	1.22×10^{-10}	8.19×10^{10}
1.25×10^{-7}	8×10^{7}	6.1×10^{-11}	1.64×10^{11}
6.25×10^{-8}	1.6×10^{8}	3.05×10^{-11}	3.28×10^{11}
3.12×10^{-8}	3.2×10^{8}	1.53×10^{-11}	6.55×10^{11}
1.56×10^{-8}	6.4×10^{8}	7.63×10^{-12}	1.31×10^{12}

APPENDIX 5A Western blot images and the antibodies employed in these studies



APPENDIX 5B Western blot images and the antibodies employed in these studies